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THE ROLE OF ESTROGEN IN THE ESTROGEN-RELAXIN RELATIONSHIP IN SYMPHYSEAL RELAXATION[1]

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EXPERIMENTAL relaxation of the pelvic ligaments was first produced in the guinea pig by injecting blood serum of pregnant rabbits into animals which were in estrus (Hisaw, 1926). It was also found that extracts of corpora lutea of the sow would elicit a similar response. Later, it was shown that castrated guinea pigs were equally responsive provided they were pretreated with estrogen, but that in animals which were not conditioned by the previous action of estrogen even very large doses of either pregnant serum or extract of corpora lutea were ineffective. The presence of a hormone which has a specific effect on the pelvic ligaments was subsequently established and named relaxin, the effectiveness of which was dependent on the previous conditioning action of estrogen on the connective tissue structures.

The determination, therefore, of the changes produced by estrogen in the tissues of the symphysis pubis is essential for a complete understanding of the relaxation reaction. Unfortunately, these effects of estrogen are not grossly obvious. No alteration of the histological appearance of the symphyseal tissues was observed following a pretreatment with sufficient estrogen to condition the symphysis to the action of relaxin. Also, the effects of estrogen seem to wear off as indicated by the fact that progressively larger doses of relaxin are required with the passing of time following a pretreatment.

This work was undertaken in order to study the action of estradiol in its conditioning process on the symphyseal tissue. While no explanation of the necessity for this pretreatment with estrogen has been determined,

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[2] Parts of this study were done as a partial fulfilment of the requirements for the degree of doctor of philosophy at Harvard University.

several physical, chemical, and histo-chemical changes have been observed and are reported in the following pages.

I. CHANGES IN WATER CONTENT OF SYMPHYSEAL TISSUES

It is customary in performing relaxin assays to pretreat castrated female guinea pigs for three or four days with estradiol in amounts varying from 0.83 μg. to 1.0 μg. daily. (This variation in time and amount is due to modifications in the routine assay procedure of different laboratories.) This pretreatment is followed by the test substance given in a single dose. The animals are then given a ten day rest period and re-used for the subsequent assay. This procedure is based on studies showing that relaxation may be induced as long as a week after cessation of estrogen treatment only by raising the minimal effective dose of relaxin fifty-fold (Abramowitz et al., 1944).

Since this sensitization with estrogen disappears gradually, the water content studies were based on groups of castrated female guinea pigs which had all received three daily doses of 1.0 μg. of estradiol[3] and the symphyses removed at stated intervals after cessation of estrogen treatment. A summary of these findings is shown in the graph of Figure 1. The major portion of these guinea pigs were animals removed from the assay colony after reaching gross weights of 600 grams. By this procedure large and easily accessible symphyseal pads were obtained. However 18 castrated animals which had had no other hormonal treatment were also used, and since the data obtained were identical, the results were included without qualification with those from the animals taken from the assay colony.

The average water content of all estrogen treated animals was $74.3\% \pm 0.55$[4] of the total wet weight. A statistically equivalent value was obtained under the following conditions:

1) Rested guinea pigs treated 3 to 6 days with 1.0 μg. of estradiol daily.
2) Guinea pigs given a single dose of estradiol (1.0 μg.) after only one week's rest from previous treatment.
3) Guinea pigs rested four weeks and given 1.0 μg. of estradiol daily for three days.

On the other hand animals killed at stated intervals after cessation of treatment showed a progressive decrease in the water content of the symphyseal pad dropping to a value of $64.7\% \pm 1.05$ of the wet weight by four weeks. (See Fig. 1.) The rapidity with which estrogen increases the water content of the pad is illustrated by a group of animals which were given a four week post-treatment rest followed by a single injection of 1.0 μg. of estradiol. This one dose raised the percentage of water in 24 hours

[3] The estrogen used in these experiments was alpha-estradiol supplied by Schering Corporation, Bloomfield, N. J.

[4] The variation factor of given values is expressed in terms of standard error.

to 70% of the wet weight. (See Table 1.) These data show, therefore, that one of the immediate effects of estrogen is to increase the water content of symphyseal tissue, and that when the symphysis is sensitive to relaxin its water content approaches 74% of the wet weight.

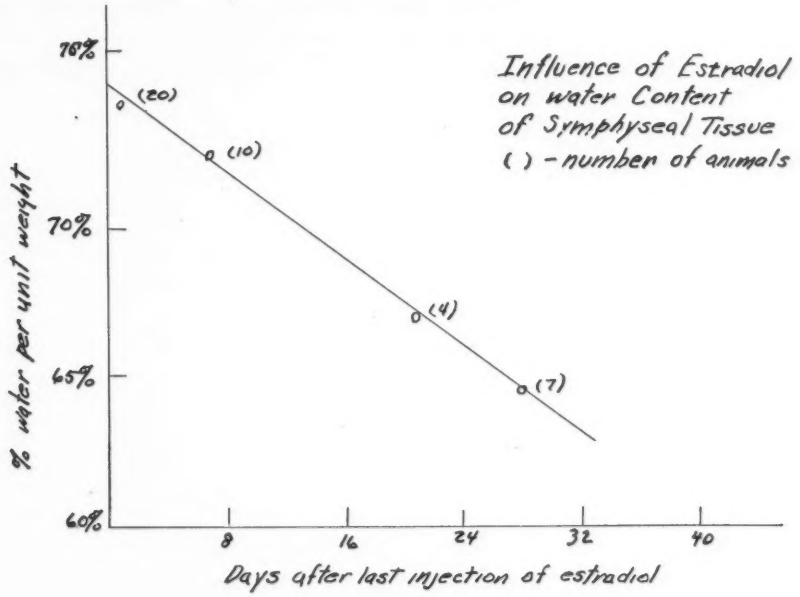

FIG. 1

In connection with this water increase, it is interesting to note that the injection of 40 G.P.U. of relaxin into pretreated animals caused an additional increase in water content, raising the percentage of water to 78.3% ± 1.0 within six hours of the injection of relaxin. This latter value is statistically equivalent to the water content of tissues from guinea pigs given twenty-five days of continuous treatment with estradiol (1.0 μg. per day). (See Table 1.)

TABLE 1. WATER CONTENT OF SYMPHYSEAL TISSUE

Number of animals	Treatment	% water per unit weight of symphyseal tissue
4	Guinea pigs given 1.0 μg. in a single dose after a four week rest from previous treatment	70.0% ± 0.80
20	Guinea pigs primed with 3 daily doses of 1.0 μg. of estradiol	74.3% ± 0.55
7	Primed Guinea pigs injected with 40 G.P.U. of relaxin	78.3% ± 1.00
4	Guinea pigs given 25 daily injections of 1.0 μg. of estradiol	80.3% ± 0.80

II. ESTROGEN STIMULATED GROWTH OF THE SYMPHYSEAL PAD

Though it has been previously noted that no gross histological changes are produced by this pretreatment with estradiol, it seemed possible that measurable growth of the tissue might still have occurred which was not discernible in histological preparations. This possibility was studied by two means; namely, by x-ray measurements of the interpubic distance before and after treatment, and by weight measurements of symphyseal pads removed at similar times.

TABLE 2. INTERPUBIC DISTANCES MEASURED BY X-RAY

Number of animals	Treatment	Interpubic distance
63	Untreated Controls*	0.33 mm. ± .001
8	Untreated Controls re-x-rayed four days later	0.37 mm. ± .052
32	Animals Primed with Estradiol 100 gram weights on legs	0.51 mm. ± .022
16	Animals Primed with Estradiol 500 gram weights on legs	0.61 mm. ± .008

* Since the results were identical, this group is a composite of those animals in which there were 100 and 500 gram weights on the hind legs.

For the x-ray studies, female guinea pigs which were castrated on attaining a weight of app. 275 grams were used when a post-operative growth to a minimum of 350 grams had occurred. By this method animals with similar histories and a minimal weight range could be used. The pictures were obtained with an ordinary dental x-ray while the animals were under ether anesthesia. The hind legs of the animals were held down by either 100 or 500 gram weights while being x-rayed.

The results of these experiments are summarized in Table 2. From these data two changes as a result of estrogen pretreatment are indicated. First, the estrogen priming caused an increase of app. 50% in the interpubic distance as based on animals x-rayed before and after treatment. Secondly, in the treated animals, the use of 500 gram weights on the legs of the guinea pigs produced an interpubic distance app. 20% greater than similarly treated animals held down with 100 gram weights. There was no statistical difference in the values obtained in a similar experiment carried out on non-treated animals. There is indicated, therefore, the possibility that elasticity has been added to the tissue as a result of the estrogen priming.

Further light on this growth induced by the estrogen pretreatment was obtained by comparing actual wet and dry weights of the symphyseal pads. To approximate similar conditions in the two series of animals, guinea pigs with identical histories were used and all were given a three day treatment with 1.0 μg. of estradiol per day. The symphyses were removed from one group at the conclusion of the pretreatment, and from the other group one week later. The data can be summarized as follows:

1) The symphyseal pads removed from six animals 24 hours after the last injection of estradiol had an average wet weight of 19.4 mg. ±3.0; an average dry weight of 5.1 mg. ±0.8.
2) The symphyseal pads removed from six animals 7 days after the last injection of estradiol had an average wet weight of 10.7 mg. ±2.0; an average dry weight of 2.9 mg. ±0.6.

The values obtained for both the dry and wet weights of the symphyseal pads removed from the two groups of animals were statistically different, thus indicating that not only does estrogen increase the water content but that actual growth takes place. Caution must be used in evaluating these figures, however, for while every effort was made to remove the entire symphyseal pad from each animal, the lesser amount of water in the rested tissue made its removal more difficult, therefore increasing the possibility of error. Their main value in consideration here is in their substantiation of the x-ray data.

III. CALCIUM AND PHOSPHORUS CONTENT OF SYMPHYSEAL TISSUE

In the normal guinea pig, the process of the development of the symphysis pubis through sexual maturity and subsequent pregnancies includes a transformation of both cartilage and bone into connective tissue. One of the physiological functions of relaxin seems to be the speeding up of this process through its ability to dissolve cartilage and connective tissue (Talmage, 1947). This process can be accomplished by estrogen alone if given in large amounts for a sufficient length of time.

TABLE 3. PHOSPHORUS AND CALCIUM CONTENT OF SYMPHYSEAL TISSUE

Mineral	Treatment	% of element per unit mass	
		of wet weight	of dry weight
Phosphorus	Average of 18 controls	0.244% ± .011	0.71% ± .032
Phosphorus	Average of 14 primed animals*	0.115% ± .017	0.45% ± .067
Calcium	Average of 18 controls	1.073% ± .156	3.09% ± .448
Calcium	Average of 14 primed animals*	2.030% ± .114	7.88% ± .441

* All treated animals were given 3 daily doses of 1.0 μg. of estradiol and the symphyses removed 24 hours after last injection.

The time and amount of hormone necessary to produce these histological changes are far too great to permit such to occur during the short pretreatment period. It nevertheless was of interest to determine whether any quantitative chemical changes in the calcium and phosphorus content of the connective tissue occur in these early stages. For these determinations two groups of animals were used. The first were female guinea pigs castrated at weights of app. 275 grams and used when reaching weights approaching 500 grams. The other group contained animals which had all had an identical previous history of estrogen administration and were

given four weeks' rest after cessation of this treatment before being used in this experiment. Quantitative determinations for calcium were made by the method of Roe and Kahn, for phosphorus by the method of Fiske and Subbarow.[5] The values obtained in both groups were statistically identical and are presented, therefore, as a single group. The dry and wet weight percentages of calcium and phosphorus are summarized in Table 3.

Examination of the data shows that even in this short period of estrogen administration there is more than a 100% increase in the calcium content per unit mass of dehydrated symphyseal tissue and a corresponding 40% decrease in total phosphorus per unit mass. Parallel studies, utilizing radiophosphorus in an attempt to determine changes in the rate of uptake of phosphorus in the two situations, showed, with the limited data available, no statistical difference in the rate of uptake of phosphorus in the control animals and those pretreated with estradiol.

IV. PHOSPHATASE ACTIVITY IN SYMPHYSEAL TISSUE

The interest in the possible influence of estrogen on the presence of phosphatase in the symphyseal tissue of the guinea pig was stimulated by previous studies (Talmage, 1949) in which estradiol was found to have a marked effect on the phosphatase in the genital tract of the rat. Tests were made, therefore, for phosphatase in the symphyseal tissue of untreated guinea pigs, in animals that had been given the regular pretreatment with estradiol (1 μg./day for 3 days), and in those in which the symphysis had been relaxed by the administration of relaxin. The qualitative and grossly quantitative presence of phosphatase was determined by the histo-chemical methods described by Gomori (1941) and by Moog (1944). Two animals were used in each category, and the experiments were repeated at a later date as an additional check. The results for both sets of experiments were identical.

Determinations for acid phosphatase proved negative in all instances, but the amount of alkaline phosphatase showed an interesting correlation with estrogenic stimulation. Very little alkaline phosphatase was detectable in the symphyseal tissue of animals that had not received estrogen for a period of two to four weeks. However, in animals under the influence of estradiol, phosphatase was present not only in the connective tissue cells but also was abundant in the collagenous matrix of the symphyseal pad. No regional difference in the amount of phosphatase could be detected as these histo-chemical tests are largely qualitative in nature, and therefore only very rough quantitative estimates could be made from the amount of precipitate laid down in the tissue.

[5] The author is indebted to Dr. Clark P. Read for his aid in the calcium and phosphorus determinations.

The duration of alkaline phosphatase in the symphysis after cessation of an estrogen treatment is rather short. The presence of the enzyme can still be detected qualitatively 96 hours after the last injection of estrogen, but by the end of one week, there was only a trace similar to that found in the untreated animal.

Relaxation of the symphysis by the administration of relaxin caused no detectable change in the amount or distribution of the enzyme throughout the symphyseal tissue. Symphyseal tissue removed, six hours, two days, and one week after the injection of relaxin showed a gradual decrease in phosphatase identical with that found in the symphyses of animals at similar periods after treatment with estradiol alone.

DISCUSSION AND CONCLUSIONS

Any attempt at an explanation for the necessity of the priming with estradiol before relaxin is able to effect the symphysis pubis of the guinea pig would at this time be highly speculative. However, that the symphyseal tissue becomes exceedingly active and that physiological changes are taking place is shown by the studies outlined above. Several pertinent correlations between the changes discussed and the sensitization process can be made.

Changes in water content of the symphyseal tissue apparently follow closely the ability of the tissue to respond to relaxin. It seems safe to assume that symphyseal tissue is not fully responsive unless the water content approximates 74% by weight, but that it remains sensitive to large amounts of relaxin when the percentage drops to the lower seventies, and presumably is insensitive whenever the water percentage falls into the sixties. Whether this phenomenon is the cause or an effect of the sensitization process can only be conjectured.

While it is possible that alkaline phosphatase may be necessary in the estradiol conditioning process of the symphysis, it does not appear that its presence in the tissue is essential for the action of relaxin, for it has been shown that symphyseal tissue of animals primed with estradiol will respond to relaxin at least a week after pretreatment, while at this time only very small amounts of the enzyme are present in the symphyseal tissue. However, it is possible that the physiological condition set up by estrogen and phosphatase may linger on for some time after both the hormone and the enzyme have disappeared from the tissues.

The immediate large increase in the calcium content of symphyseal tissue and the corresponding decrease in total phosphorus proved to be very surprising and cannot with present information be correlated with the sensitization process. However, these results are further confirmation that estrogens readily enter into the metabolism of supportive tissues, being concerned possibly with basic enzymatic processes.

Finally, the studies on the growth of the symphyseal pad produced by the estrogen pretreatment indicate that this connective tissue is very sensitive to estrogen. As a result of its stimulation numerous metabolic changes appear to occur in the tissue, any or all of which may be necessary before relaxin can produce its effect.

SUMMARY

The studies described in the preceding pages deal with physical, chemical, and histo-chemical changes produced in the symphysis pubis of the guinea pig as a result of a priming treatment with estradiol which consisted of three daily doses of 1.0 g. These may be summarized as follows:

The water content of symphyseal tissue is raised to 74.3% of its total weight as a result of the estrogen treatment. This decreases slowly after cessation of treatment dropping to 64.6% after 28 days.

The interpubic distance as measured by x-ray is, after the three day treatment, approximately 50% greater than in the non-treated condition. This growth of the symphyseal pad is correlated with results which indicated that the total dry weight of the symphyseal pad as well as the total wet weight increases statistically during the priming process.

Another marked and surprising effect of estrogen during this short period is to increase the total calcium per unit dry weight of tissue app. 100% while decreasing the total phosphorus app. 40%.

The last change discussed is the increase in the alkaline phosphatase from almost negligible amounts in the controls to a quantitatively heavier concentration both in the connective tissue cells and the collagenous matrix of the symphyseal tissue of the treated animals.

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DECREASE IN PLASMA LEVELS OF ENDOGENOUS PROGESTERONE DURING RENAL PASSAGE[1]

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IT SEEMS established that two of the end products of the metabolism of progesterone in certain species are pregnanediol-3(α),20(α) (Venning and Brown, 1937; Westphal and Buxton, 1939a; Heard, Bauld, and Hoffman, 1941; Westphal, 1942) and pregnanol-3(α)-one-20 (Dorfman, Ross, and Shipley, 1948). Whether other compounds are also end products is not definitely known, nor is it known where in the body the conversion occurs. Ovaries, uterus, placenta, and testis are not essential for the transformation (Westphal and Buxton, 1939a; Venning and Brown, 1940; Hamblen, Cuyler, and Hirst, 1940; Buxton, 1940; Heard, Bauld, and Hoffman, 1941; Hoffman, 1942), although it may occur in each of them.

The two organs thus far implicated in the inactivation of progesterone are the liver and the kidney. Several authors have reported inactivation of progesterone by the liver *in vivo* (Selye, 1941; Kochakian, Haskins, and Bruce, 1944; Dosne, 1944; Masson and Hoffman, 1945; Engel, 1946; Forbes and Hooker, 1949). Hepatic inactivation is reported not to occur *in vitro*, however (Zondek, 1939; Engel, 1944). The nature of the hepatic inactivation is not known, although pregnanediol-3(α), 20(β) and pregnanol-3(α)-one-20 have been isolated from bile of pregnant cows (Pearlman and Cerceo, 1948). In the mouse hepatic inactivation appears to involve binding or conjugation of progesterone (Forbes and Hooker, 1949); other possibilities were not examined.

Evidence that the kidney is capable of inactivating progesterone has been reported by Kochakian, Haskins, and Bruce (1944), who implanted pellets of progesterone into the kidneys of suitable rabbits. The inactivation observed was distinctly less than that in the liver. The ability of the kidney to inactivate other steroid hormones has been shown by incubation of estrogens with kidney tissue (Heller, 1940), by incubation of an andro-

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gen with kidney tissue (Samuels, 1949), and by perfusion of kidney with estrogens (Schiller, 1945) and androgens (Danby, 1940).

The foregoing studies have been concerned only with artificially supplied progesterone. The availability of a sensitive and apparently specific bioassay for progesterone (Hooker and Forbes, 1947, 1949a) that permits measurement of blood levels (Hooker and Forbes, 1949b) has made it possible to ascertain *in vivo* whether renal passage affects levels of endogenous progesterone in blood.

EXPERIMENTAL

The animals studied were monkeys in the latter one-half of the menstrual cycle and pseudopregnant rabbits. These animals were selected because, among other reasons, the rabbit can convert progesterone into pregnanediol (Heard, Bauld, and Hoffman, 1941; Westphal, 1942), while the monkey apparently cannot (Westphal and Buxton, 1939b; Marker and Hartman, 1940). Seven adult, virgin rabbits were given an intravenous injection of gonadotrophin[3] and blood samples were drawn six or seven days later. Pseudopregnancy was verified by inspection of the ovaries and, in some instances, by microscopic study of the endometrium. The day of the menstrual cycle of the monkeys was based upon records of daily vaginal smears.

Under Nembutal (monkeys) or Nembutal and ether (rabbits) anesthesia, 1 ml. samples of blood were aspirated first from a renal vein and then from a renal artery. In rabbit 234 the vein and artery employed were contralateral; in all other instances the vessels used supplied the same kidney. An effort was made to keep to a minimum the interval between the drawing of the two samples in each animal; the actual interval ranged from 4 to 24 minutes, depending upon the difficulties encountered. In all instances sodium citrate served as the anticoagulant. Urine was aspirated through the bladder wall (2 rabbits, 1 monkey) or collected as voided (one rabbit) at the time of drawing the blood samples. Urine from two monkeys was collected in metabolism cages.

The blood samples were centrifuged promptly, and the plasma progesterone was separated into its free and bound fractions by a modification (Hooker and Forbes, 1949b) of the method employed by Szego and Roberts (1947) for the separation of free and protein-bound estrogen in blood. Each fraction was incorporated in sesame oil for assay.

Essentially the same procedure was followed in attempting to separate free and bound progesterone in the urine. A measured volume of each specimen was added drop by drop with stirring to ten volumes of cold acetone, and the precipitate was allowed to settle at least overnight in a refrigerator set at 3°C. The supernatant, assumed to contain the free progesterone, was removed after centrifugation. The precipitate was washed three times with several volumes of a cold 1:1 ether-acetone mixture, and centrifuged after each washing. The supernatant and washings were combined, and the ether and acetone were removed by distillation under diminished pressure. The aqueous residue, representing the original water of the urine, was extracted five times with ether. A measured volume of sesame oil was added to the ethereal extract, and the ether was removed by distillation.

The aqueous residue of the urine of rabbit 234 was acidified to pH 1 and heated for

[3] The gonadotrophins employed were Synapoidin, supplied through the courtesy of Drs. D. A. McGinty and Oliver Kamm of Parke-Davis Company, and Pituitary Gonadotrophin, kindly furnished by Dr. H. Sidney Newcomer of the Squibb Company.

six hours in a boiling water bath. The residue was again extracted with ether and the dissolved material was incorporated in sesame oil.

The material precipitated by the acetone was allowed to dry in air at room temperature, after which it was powdered and suspended in a small volume of water. The suspension was acidified to approximately pH 1 with hydrochloric acid and immersed in a boiling water bath for six hours. The material thus partially hydrolyzed was extracted five times with ether, and the material extracted by ether was transferred to sesame oil as described above. This fraction was considered to be the bound progesterone.

All of the oily specimens were bioassayed for their progesterone content in our usual manner (Hooker and Forbes, 1947), and the original concentrations in plasma and urine were calculated.

RESULTS

The values given by the assays are listed in Table 1. It will be seen that in every instance the plasma concentration of free progesterone in the renal

TABLE 1. PLASMA AND URINE PROGESTERONE LEVELS, μg./ml

Animal	Status	Free progesterone			Bound progesterone		Urinary progesterone	
		Renal artery	Renal vein	Decrease	Renal artery	Renal vein	Free	Bound
Rabbit 5	7 days pseudopreg.	7.0	1.5	79%	0.3	0.8		
Rabbit 4	7 days pseudopreg.	6.9	5.8[6]	16%	0.39	0.19		
Rabbit 2	7 days pseudopreg.	5.5	2.9	47%	<0.2	<0.2		
Rabbit 10	7 days pseudopreg.	4.7	1.4	70%			<0.05[1]	
Rabbit 234	6 days pseudopreg.	4.6	3.3[4]	29%	0.14	0.15	0.01	0.01[2]
Rabbit 1	7 days pseudopreg.	3.6	0.2	94%	<0.2	0.2		
Rabbit 9	7 days pseudopreg.						0.4[2]	
Monkey 1762	Day 24 (34-day cycle)	7.7-8.9[4]	2.6	69%	0.2	0.2		
Monkey 1811	Day 25 (28-day cycle)	2.8[5]	2.0	29%	0.25	<0.25		
Monkey 1608	Day 24 (26-day cycle)	0.9	0.2	78+%	0.2	<0.2	0.006	<0.003[3]
Monkey 1762	Day 26 (42-day cycle)						<0.01	<0.013[7]
Monkey A-11	Day 3 (34-day cycle)						0.2	0.07[2]

[1] Freshly voided urine.
[2] Bladder urine.
[3] Collected from metabolism cage.
[4] Incomplete assay.
[5] Plasma from adjacent portion of aorta assayed at 2.7 μg./ml. free, and 0.25 bound progesterone.
[6] Sample may have been contaminated with arterial blood.
[7] 45-hour postoperative specimen.

vein was less than that in the renal artery, the decrease ranging from 16 to 94 per cent for the rabbits and from 29 to 78+ per cent for the monkeys. The wide range in differences may have been due, at least in part, to the fact that the two samples from a given animal were not obtained simultaneously. The magnitude of the difference, however, was not obviously correlated with the time elapsed between the drawing of the two samples. The smallest arterio-venous differences seen in the rabbits were not obtained under ideal circumstances and may be open to question. The venous sample from rabbit 4 may have been contaminated with arterial blood, and the two samples from rabbit 234 were obtained from contralateral vessels. The extent of the decrease in free progesterone seems unrelated to the arterial level.

The values for bound plasma progesterone seem to follow no pattern.

In three instances the venous level was lower than the arterial level, in three instances the reverse was found, and in two animals the levels were identical or were too low to measure.

The urinary concentration of both free and bound progesterone was low in all of the specimens studied, but wide differences were found from animal to animal.

DISCUSSION

The striking difference between the concentrations of free plasma progesterone in the renal artery and vein shows that renal passage removed free progesterone from the blood. The decrease in level could have been accomplished by: (1) renal excretion of unchanged progesterone; (2) conversion of active progesterone into an inactive form; or, (3) conversion to some other compound which is then excreted or carried into the venous system.

The extremely low urinary concentrations seem to exclude excretion of unchanged progesterone as the explanation of the renal arterio-venous differences.

One means of conversion of active progesterone to an inactive form is binding to protein or conjugation, inasmuch as bound progesterone is biologically inactive (Hooker and Forbes, 1949b; Forbes and Hooker, 1949). The venous level of bound progesterone, however, was equal to or lower than the arterial concentration in five animals and but slightly higher in the other three animals. The three increases are altogether inadequate to account for the free progesterone lost. The urinary concentrations of bound progesterone also appear to be much too low to account for the loss of free progesterone from the plasma.

The most likely possibility seems at the moment to be conversion of free progesterone into another compound. Pregnanediol is a possible product in the rabbit, which can convert injected progesterone into pregnanediol (Heard, Bauld, and Hoffman, 1941; Westphal, 1942). If pregnanediol is the product formed from endogenous progesterone in the kidney, it is excreted in quantites too small to be detected by the usual methods in both the monkey (Westphal and Buxton, 1939b; Marker and Hartman, 1940) and the rabbit (Westphal and Buxton, 1939b). The quite incomplete recovery of sodium pregnanediol glucuronide in urine after this substance is injected intravenously in rabbits (Hoffman, 1942) may be significant in relation to the present problem.

The plasma level of free progesterone may be regarded as the net balance of progesterone released by the corpora lutea and possibly other sources minus progesterone removed, transformed, or bound by the kidney, the liver, and other organs or tissues. If a steady plasma level is to be main-

tained for a period of time, the amount of progesterone added to the plasma during that period must equal the amount removed during the same interval. For the purpose of illustration, it may be supposed that in a monkey the renal arterial concentration of free progesterone is 6 μg./ml, that a volume of blood containing 300 ml. plasma, and hence 1.8 mg. progesterone, circulates through the two kidneys in two minutes, and that 66 per cent of the free hormone is removed by renal passage. Under these circumstances renal passage would remove 66 per cent of 1.8 mg., or 1.2 mg., of free progesterone during this period, or 0.6 mg./min. It also follows that 0.6 mg. free progesterone/min. must be added to the plasma from some source to compensate for the renal loss alone.

If all of the progesterone being added to the blood according to this schedule is newly formed (secreted), the rate of luteal production of progesterone is enormously higher than is generally believed. It will be recalled that an extremely rapid rate of secretion of adrenal cortical hormones has been shown by Vogt (1943).

The amount of progesterone that must be formed in one minute according to these computations, indeed, approximates the total daily subcutaneous dose of progesterone that will provoke and maintain premenstrual changes in the endometrium of the monkey (Allen, Hisaw, and Gardner, 1939). This circumstance suggests that much of the progesterone inactivated by the kidney may not be irretrievably lost, i.e., that renal alteration of progesterone may be reversible. The compound formed in the kidney may be reconverted into active progesterone in some other organ(s). In this event, the rate of new synthesis of progesterone need not occur nearly so rapidly. If the compound formed in the kidney is a reduced form of progesterone (that is, one or another stage in the formation of pregnanediol), recoversion into progesterone is the reverse process, oxidation, and in this respect is like the presumed synthesis of progesterone in luteal tissue.

The low values for arterial, venous, and urinary concentrations of bound progesterone raise questions. Evidence has been presented (Forbes and Hooker, 1949) that in the mouse bound plasma progesterone is biologically inactive and that binding is a mechanism of hepatic inactivation. If these circumstances obtain in other species, the low levels of bound progesterone found under all physiologic conditions studied thus far suggest an efficient mechanism for preventing accumulation of this fraction. The urinary levels of bound progesterone seen in the present experiment suggest that renal excretion is not the mechanism.

SUMMARY

Blood samples of approximately 1 ml. were drawn successively from the renal vein and artery of each of three rhesus monkeys in the latter one-half

of the menstrual cycle and of six pseudopregnant rabbits. Bio-assay revealed the plasma concentration of free progesterone in the vein to be 16 to 94 per cent less than in the artery. The level of bound progesterone did not appear to be significantly or consistently affected by renal passage. The concentrations of free and of bound progesterone in urine specimens from six animals were much too low to account for the loss of free progesterone in the plasma.

Computations based upon a typical renal loss suggest that renal inactivation of progesterone may occur at a rate on the order of 0.6 mg. per minute. Maintenance of the arterial level would apparently necessitate secretion of the hormone at the same rate or re-activation of much of the inactivated progesterone.

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THE METABOLISM OF TESTOSTERONE BY LIVER TISSUE OF NIACIN, TRYPTOPHAN OR THIAMINE DEFICIENT RATS[1]

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THE role of the vitamins in the metabolic inactivation of hormones by the liver has been investigated by Biskind (1943) using the splenic implantation of the hormone in rats on deficient diets fed *ad libitum*. In such animals, however, there is a loss of appetite and, therefore, multiple deficiencies develop. Drill and Pfeiffer (1946) have shown by biological techniques that this is a major factor in the reduced estrogen inactivation following thiamine deficiency. The present series of experiments was undertaken, therefore, to attempt to determine the relative role of certain water-soluble vitamins in testosterone metabolism under conditions where the intake of other dietary constitutents was maintained.

Niacin deficiency was chosen because of the evidence already obtained that diphosphopyridine nucleotide (DPN) is an essential cofactor in the enzymic reaction whereby an alcohol group on carbon-17 of testosterone is oxidized to a ketone (Sweat and Samuels, 1948). To achieve niacin deficiency rapidly it was also necessary to remove tryptophan since it had been demonstrated that the rat synthesizes niacin from this amino acid (Rosen, Huff and Perlzweig, 1946). Parallel controls with tryptophan deficiency alone were obviously essential. Thiamine deficiency was studied because of its disputed role in estrogen inactivation, although declared by Biskind (1943) to be nonessential for testosterone metabolism.

EXPERIMENTAL

Adult male rats of the Sprague Dawley strain were maintained on a diet of Purina fox chow before the experiments began. They were then placed on the diets given in Table 1 as indicated in the description of each experiment. At the end of the feeding period the rats were killed by decapitation and allowed to bleed freely. The livers were removed, minced and incubated with testosterone as described previously (Samuels, 1947, Samuels, McCaulay and Sellers, 1947, Sweat and Samuels, 1948). The testosterone

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TABLE 1. COMPOSITION OF DIETS

Constituent	Diet I	Diet II	Diet III	Diet IV	Diet V	Diet VI
	gm.	gm.	gm.	gm.	gm.	gm.
Zein	17.55	17.55	17.55	17.55		
Casein					20.8	20.8
Sucrose	65.12	65.12	65.12	65.12	58.3	58.3
Corn Oil	12.78	12.78	12.78	12.78	16.8	16.8
Salt Mixture No. 2	4.00	4.00	4.00	4.00	4.0	4.0
Oleum Percomorphum (Mead's)	0.10	0.10	0.10	0.10	0.1	0.1
l-lysine	0.40	0.40	0.40	0.40		
dl-tryptophan	0.15			0.15		
	mg.	mg.	mg.	mg.	mg.	mg.
Thiamine Chloride	0.20	0.20	0.20	0.20	0.20	
Riboflavin	0.30	0.30	0.30	0.30	0.30	0.30
Calcium Pantothenate	2.00	2.00	2.00	2.00	2.00	2.00
Pyridoxine HCl	0.25	0.25	0.25	0.25	0.25	0.25
Choline Chloride	100.00	100.00	100.00	100.00	100.00	100.00
Inositol	10.00	10.00	10.00	10.00	10.00	10.00
Biotin	0.01	0.01	0.01	0.01	0.01	0.01
Para-amino Benzoic Acid	10.00	10.00	10.00	10.00	10.00	10.00
Vitamin K	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin E	5.00	5.00	5.00	5.00	5.00	5.00
Niacin	10.00		10.00		10.00	10.00

1 gm. of dry diet is equivalent to 4.51 calories.

Stomach Tube Diets: To each 100 gm. of the above diets 50 ml. water were added making a total of 120 ml. Liquid diets are designated Ia, IIa, IIIa, IVa, Va, and VIa. Rats were fed 8 ml. twice daily, equivalent to 59 calories per day.

was determined spectrophotometrically using the 240 mμ absorption band due to the α-β unsaturated ketone structure. An aliquot representing 20% of the extract was also used for determination of 17-ketosteroids by the method of Callow, Callow and Emmens (1938).

In experiment 1 three groups of rats of similar age were force-fed by stomach tube diets Ia, IIa and IIIa, Table 1. These were the control diet, niacin-tryptophan-deficient diet, and tryptophan-deficient diet respectively. The rats were fed 8 ml. twice daily which was equal to 59 calories per animal per day. The feeding technique was that of Reinecke *et al.* (1939).

The excretion of N-methylnicotinamide was determined on a 24-hour collection of the pooled urines from each of the different groups. The analyses were carried out by the method of Huff and Perlzweig (1947). After 17 days of feeding, analysis of the urine for N-methylnicotinamide showed that the animals on the deficient diets were excreting decreased amounts of this compound. The rats were then killed and the incubations with liver tissue carried out. Aliquots of the livers used for the metabolic studies were also analyzed for total niacin (nicotinic acid plus nicotinamide) by the method of Goldthorpe (1950).

In experiment 2 four groups of rats were fed diets I, II, III, and IV (Table 1) *ad libitum* for 72 days. Analysis of the urine for N-methylnicotinamide was carried out at frequent intervals in order to follow the developing tissue deficiency. On the 72nd day the animals were killed and the ability of the minced liver to metabolize testosterone was determined as above. Samples of liver tissue were also analyzed for total niacin.

In experiment 3 diets V and VI were fed. After 19 days the animals were killed and the incubation experiments carried out as previously described.

Experiment 4 was the same as experiment 3 except that in addition to the tissue with buffer solution alone, suspensions to which 0.001 M diphosphopyridine nucleotide or 0.001 M citrate were added were also used.

RESULTS

In experiment 1 (Fig. 1) the rate of destruction of testosterone by the liver tissues of force-fed deficient rats was compared with that of similarly fed controls. The rate was lower in the tryptophan-deficient animals

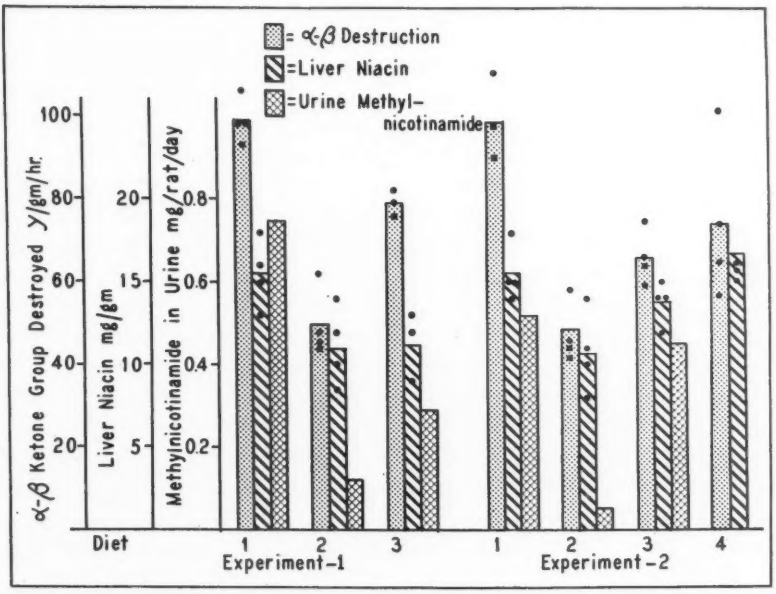

FIG 1. The Metabolism of Testosterone by Liver Tissue of Rats on Diets Deficient in either Tryptophan, Tryptophan and Niacin, or Niacin.

A known weight of minced tissue in the range of 0.60 – 1.00 gm. was incubated with 200γ testosterone and 25 ml. of a buffer solution containing 0.0056 M. KCL, 0.0021 M $MgCl_2$, 0.08 M NaCl, 0.04 M Na_2HPO_4—NaH_2PO_4 buffer at pH 7.4. Incubation was for one hour in O_2 atmosphere at 38°C. Diet 1: complete; diet 2: tryptophan-niacin deficient; diet 3: tryptophan-deficient; diet 4: niacin deficient. Each spot represents the mean of duplicate determinations on a single liver. Height of the bars represents the group mean.

but the difference was not marked. The rate of destruction of testosterone by the liver tissue of rats force-fed a diet deficient in both niacin and tryptophan was only about half as great as the controls. This was significantly lower than when tryptophan alone was absent from the diet. The recoveries of testosterone in the boiled samples in both groups were good. Samples from the same rat liver were also in close agreement in most cases.

A deficiency of both niacin and tryptophan in the animal apparently markedly decreases its ability to metabolize testosterone.

In experiment 2 diets deficient in both niacin and tryptophan, niacin alone, and tryptophan alone were fed *ad libitum*. As in the force-fed experiments the tissues of the niacin-tryptophan-deficient rats showed a rate of testosterone destruction about half that of the control group.

In the niacin-deficient group there was one rat which showed a rate of destruction equal to that of the control animals. Perhaps this animal had greater stores of niacin or had the ability to synthesize a larger amount from the ingested tryptophan. The rest of the livers from animals on diets deficient in only one of the two components studied showed a reduced ability to metabolize testosterone; it was not as marked, however, as when both niacin and tryptophan were absent from the diet. A comparison of the activities of the livers from the tryptophan-deficient rats (diets III and IIIa) in the two experiments indicates a significantly greater reduction in activity in the tissues from the *ad libitum*-fed animals. This is probably due to the general effect of the reduced food intake of this group. As previously shown (Sweat and Samuels, 1948), rats which are fasted rapidly lose the ability to destroy the α-β conjugated ketone group in testosterone.

The enzymic activity of the tissues from control and doubly deficient rats respectively were similar in the two experiments. Since the food intake of the deficient rats markedly decreased in experiment 2 while it was artificially maintained in experiment 1, it would appear that the removal of both niacin and tryptophan was the primary factor in the marked reduction in enzyme activity seen in these groups and not the decreased intake of other factors.

No significant amounts of 17-ketosteroids were found in any of the experiments.

The decrease in total niacin content of the livers in both experiments was not as marked as the decrease in testosterone destruction. Only in the case of the doubly deficient animals was there a significant fall. Individual variations in steroid metabolic activity and niacin content within groups showed no correlation.

The N-methylnicotinamide excretion was correlated better with testosterone destruction among groups. Since the excretion of individual rats was not measured the correlation within groups could not be determined. It would seem, however, that the change in DPN available for testosterone metabolism lay somewhere between levels indicated by the total niacin content of the livers and those represented by the excretion of the methylated derivative.

In experiment three (Fig. 2) the destruction of testosterone by the livers of the force-fed thiamine-deficient rats was compared with that of the con-

trols, no cofactor being added to either. There was a reduced rate similar to that found with tryptophan deficinecy; it was not so marked, however, as in the absence of both tryptophan and niacin.

These observations were confirmed in experiment 4. It was also shown that addition of DPN gave rates similar to that of normals with this co-

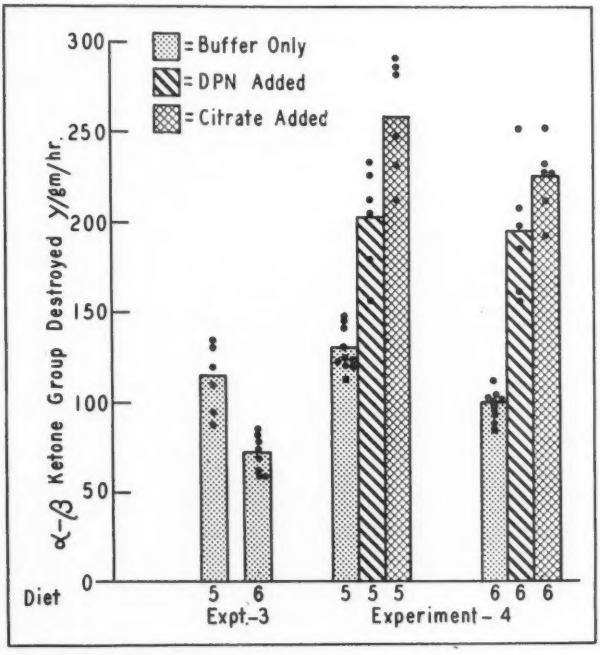

FIG. 2. Metabolism of Testosterone by Liver Tissue of Thiamine-Deficient Rats with and without Added Cofactors.

A known weight of minced tissue in the range of 0.60 – 1.00 gm. was incubated with 200γ testosterone and 25 ml. of a buffer solution containing 0.0056 M KCL, 0.0021 M $MgCl_2$, 0.08 M NaCl, 0.04 M Na_2HPO_4—NaH_2PO_4 buffer at pH 7.4. Incubation was for one hour in O_2 atmosphere at 38°C. Cofactors were added in concentration of 0.001 M. Diet 5: complete; diet 6: Thiamine-deficient.

factor added. When citrate was added, however, the difference between the two diets appeared to remain, although the increase in the ability of the liver tissue to destroy testosterone was similar in both groups. The deficiency, therefore, seemed to be in DPN.

These rats, like others previously studied, showed marked distention of the stomach when force-fed the thiamine-deficient diet for this length of time. The distention is apparently due to loss of gastric and intestinal mus-

cle tone. As a result, the amount of food in the gut was about equal to two days' food supply. The animals, therefore, had been absorbing decreasing amounts of food over several days and were in negative nitrogen balance and general vitamin deficiency of mild degree. The reduction in the ability of the livers to metabolize the α-β ketone group was probably due to this effect rather than thiamine deficiency directly.

DISCUSSION

As Sweat and Samuels (1948) have shown, both diphosphopyridine nucleotide (DPN) and citrate are involved as cofactors in the metabolism of testosterone by liver tissue. The step involving DPN is an oxidation of the alcohol group on carbon-17. The androstenedione so formed then appears to undergo reduction in ring A through the activity of another enzyme. A third enzyme, apparently requiring citrate as a cofactor, can act directly on the conjugated system in ring A of testosterone (Levedahl and Samuels, 1950). Whether the change produced in ring A by the last two enzymes is the same remains to be determined, but the action of each will decrease ultraviolet absorption at 238 mμ. Since niacin is a constituent of the DPN molecule a deficiency of this vitamin should lead to a reduction in the activity of the oxidative enzyme acting on carbon-17. This, in turn, would prevent the reduction of ring A by the enzyme acting on the diketone. This conclusion seems to be borne out by the present experiments.

Niacin deficiency, however, as Rosen, Huff and Perlzweig (1946) have pointed out, cannot be produced as rapidly in the presence of high tryptophan as in its absence, since tryptophan is converted into niacin. It is not surprising, therefore, that the tissues of the rats on the doubly deficient diet were less able to metabolize testosterone than those from either deficiency alone. The order, although not the magnitude, of reduction in enzymic activity is paralleled by the decrease in N-methylnicotinamide excretion.

Two possible explanations of the greater decrease in hepatic enzymic activity than in niacin content are worthy of consideration. The first is that the DPN available for the reaction was decreased more than the total niacin. According to Handler and Dann (1941) 58% of hepatic nicotinic acid in rats on a normal diet is free. An increase in this percentage would account for the results noted here. Anderson, Teply and Elvehjem (1944) report, however, that on deficient diets the decrease in coenzyme I content of the liver parallels the decrease in niacin content. This would not be in accord with this hypothesis.

The more probable explanation is that deficiency in tryptophan led to a decreased synthesis of the enzyme. In the doubly deficient group, therefore, two factors were acting to decrease the rate of reaction: decreased

enzyme and decreased electron acceptor. In the tryptophan-deficient groups the enzyme was probably the limiting factor.

Since no 17-ketosteroids were found, the step of the metabolic system in which these disappear must not have been decreased more by either tryptophan or niacin deficiency than the decrease in the oxidative step. These compounds, therefore, do not seem to be more important in the subsequent metabolic processes than in the DPN-catalyzed reaction. This would be expected in the case of niacin but could not be predicted in tryptophan deficiency.

The results with thiamin would tend to confirm Biskind's (1943) observation that this vitamin is not essential for testosterone metabolism. It does indicate, however, that the indirect effect on absorption of food, and in the case of the animal fed *ad libitum*, on appetite, may make significant changes in the rate of destruction of testosterone. This is apparently associated with decreased availability of DPN. The reasons why a general deficiency should interfere so soon with the availability of DPN for this reaction is being investigated further. Thiamin does not seem to play a specific role in the destruction of the α-β ketone group of testosterone, but does cause a significant drop in the rate of destruction through its influence on intestinal motility and absorption.

SUMMARY

A deficiency of both niacin and tryptophan in the diet decreased the destruction of testosterone by the liver tissue of rats to about one-half that of the controls.

A deficiency of either tryptophan or niacin alone in the diet decreased the ability of rat liver tissue to destroy testosterone, but by a much smaller proportion.

Niacin is apparently important because it is a constituent of diphosphopyridine nucleotide, which is a cofactor in one of the enzymic reactions involved in testosterone metabolism.

Tryptophan seems involved in the metabolism of testosterone because of its synthesis into niacin by the rat. It may also be an essential constituent of the enzyme.

Thiamine deficiency also produced a small decrease in the rate of destruction of testosterone by the liver tissue of force-fed rats. This appeared to be due to a secondary deficiency of DPN because of the failure of food transport and absorption in the gut.

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DIRECT ACTION OF MALE SEX HORMONE ON THE ADRENAL CORTEX[1]

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THE interrelationship between the adrenals and the reproductive system has been well studied, experimentally as well as clinically, as regards the influence of the adrenals on the sexual organs. The other aspect of the problem, that is, the effect of the gonads on the adrenals, has not been given as much attention. The action of the androgenic substances on the adrenals seems, in particular, somewhat obscure and contradictory.

In 1938 Cutuly and McCullagh noted that in hypophysectomized rats an increase of the adrenals resulted from injections of testosterone propionate. They thought that "the unexpected high weight of these glands was probably due to variable quantities of lipoidal material since it has been demonstrated that the medulla does not change greatly in weight after hypophysectomy." In 1943 Leathem and Brent showed that there was a decreased rate of atrophy of the cortex in hypophysectomized male rats if androgens were injected. In 1944 Leonard, studying the effect of various androgenic substances on the adrenal cortex of hypophysectomized rats, obtained the same kind of results as the previous authors, and attributed this to the unchanged state of the cell size.

The maintenance of the adrenals by androgens seemed to be definitely established by the previous works until the recent study of Lewis, de Majo and Rosemberg (1949) showed that vinyl-testosterone as well as testosterone propionate decreased the weight of the adrenals in normal, castrated and hypophysectomized rats.

It appeared therefore, that a new study of this controversial problem would not be devoid of interest. Three types of experiments have been devised: first, testosterone propionate was injected into hypophysectomized female rats; second, the same substance was given to hypophysectomized male rats; third, testosterone propionate was given to hypophysectomized-gonadectomized male rats, in order to see if the action of the male sex hormone on the adrenals was a direct one.

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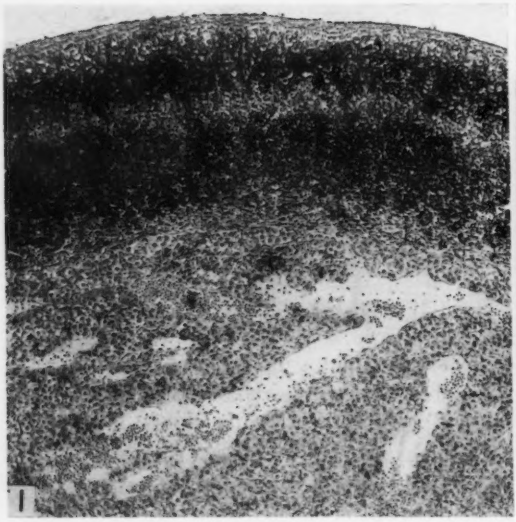

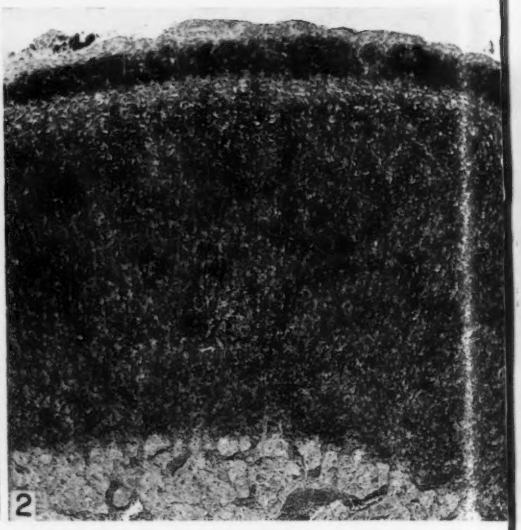

Adrenals of hypophysectomized 40 day old male rats, 15 days postoperative. Sudan orange ×83.
FIG. 1: uninjected control.
FIG. 2: testosterone propionate 2.5 mgm. daily for 15 days subcutaneously.

I. TESTOSTERONE PROPIONATE IN HYPOPHYSECTOMIZED FEMALE RATS

In the first series of experiments the possibility of repair by testosterone propionate of the adrenals of the hypophysectomized female rat after a period of regression was investigated. Female rats, hypophysectomized at 25–28 days of life were injected beginning 14 days after the operation. Testosterone propionate[2] was given subcutaneously for 10 days at daily doses of 2.5 mgm. As usual in the adrenal repair test in this type of animal, adrenal weights are of little or no value so that the results are entirely dependent upon the histological pattern. Partial repair was observed; the cortex did not show any enlargement. The glomerular zone was unchanged, the subglomerular lipid free zone was infiltrated by very fine lipid particles. In the zona fasciculata and zona reticularis, the droplets were not as coarse as in the control animals. The action on the thymus was significant since a great decrease of the weight of this organ appeared at autopsy (72 mgm. in injected animals, 115 in controls). There was therefore only a slight repair from the androgenic substance on the adrenals of hypophysectomized female rats.

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[2] The material used was "Oreton" brand of testosterone propionate for which we wish to thank the Schering Corporation, Bloomfield, New Jersey.

II. TESTOSTERONE PROPIONATE IN HYPOPHYSECTOMIZED MALE RATS

A second group of experiments was performed on male rats, hypophysectomized at 40–42 days of age. Subcutaneous injections were begun on the day of operation and continued for 15 days, at daily doses of 2.5 mgm.; the rats were, therefore, 55 to 57 days old at the time of autopsy.

The adrenals were, at autopsy, more than twice as large as those of the hypophysectomized controls (19.6 mgm. in the injected animal—8.8 in the control group). The adrenal cortex of the injected rats was much wider

TABLE 1. ACTION OF TESTOSTERONE PROPIONATE ON HYPOPHYSECTOMIZED FEMALE RATS
26–28 days old at operation; injections from 14 to 24 days p.o.

Group	Body weight		Adrenal weight (mgm.)	Thymus weight (mgm.)
	Operation (gm.)	Autopsy (gm.)		
Testosterone propionate (2.5 mgm. daily)	71	71	8	69
	68	61	8	21
	76	81	11	73
	69	61	6	150
	65	55	5	21
	71	80	9	98
	Av. 70	68.1	7.8	72
Uninjected control	62	62	9	125
	69	69	10	133
	70	54	7	123
	67	61	8	123
	80	79	7	126
	63	58	8	64
	Av. 68.3	63.8	8.1	116

than that of the uninjected control. The frozen sections of the adrenals stained for lipid (sudan orange) demonstrated the maintenance even more clearly. The cell size and the amount and distribution of cortical lipids resembled much more closely the normal than the hypophysectomized condition. The lipid droplets were fine as in normal and evenly distributed throughout the zona fasiculata and reticularis, contrasting with the coarse and irregular distribution of the same particles in the uninjected controls. The glomerular zone was practically unchanged (lipid coarse and dark staining); the subglomerular sudanophobe zone showed slight infiltration with very fine lipid droplets.

The action on the thymus was here, too, of interest, since evidence was given of a great reduction in the weight of this organ (89 mgm. in injected animals, 188 mgm. in the uninjected controls).

It might be said that there was clear evidence of maintenance of adrenal weight and lipid pattern.

TABLE 2. ACTION OF TESTOSTERONE PROPIONATE ON HYPOPHYSECTOMIZED MALE RATS
40–42 days old at operation; injection from 1 to 15 days p.o.

Group	Body weight		Adrenal weight (mgm.)	Thymus weight (mgm.)
	Operation (gm.)	Autopsy (gm.)		
Testosterone propionate (2.5 mgm. daily)	118	122	23	70
	131	124	20	70
	113	120	20	110
	125	124	14	126
	127	120	20	54
	119	124	21	105
	Av. 122	122	19.6	89
Uninjected control	120	108	10	174
	119	118	6	82
	118	113	10	230
	163	146	9	190
	125	124	8	194
	130	120	10	269
	Av. 129	120.5	8.8	188

III. TESTOSTERONE PROPIONATE IN HYPOPHYSECTOMIZED-GONADECTOMIZED RATS

A third group of experiments was performed on hypophysectomized and gonadectomized male rats in order to see if the action of the male hormone on the adrenals was a direct one and not secondary to its action on gonads.

Male rats were gonadectomized at 26 days of age, hypophysectomized ten days later and subcutaneous injections of 2.5 mgm. of testosterone propionate were given on the day of operation and continued daily for 15 days, as in the preceding experiments. The adrenal weights were markedly

TABLE 3. ACTION OF TESTOSTERONE PROPIONATE ON HYPOPHYSECTOMIZED GONADECTOMIZED MALE RATS

Gonadectomized at 26 days, hypophysectomized at 40 days; injections from 1 to 15 days p.o.

Group	Body weight		Adrenal weight (mgm.)	Thymus weight (mgm.)
	Operation (gm.)	Autopsy (gm.)		
Testosterone propionate (2.5 mgm. daily)	130	134	17	66
	134	146	21	72
	112	120	16	65
	126	136	16	84
	112	116	14	73
	130	114	12	68
	Av. 123	127	18	71
Uninjected control	117	114	12	131
	118	106	9	236
	122	110	7	244
	142	122	13	253
	112	106	8.5	224
	116	110	10.5	230
	Av. 121	111	10	219

larger than in the controls (16 mgm. in the experimental animals, 10 in the uninjected controls).

The cortex was wider than in the operated control; cell size was increased. The lipid distribution was practically normal for the male. The zona fasciculata and the zona fascicularis were wider, too, than in the control and infiltrated with fine lipid droplets.

The testosterone propionate had a marked effect on the thymus which was greatly decreased, weighing 71 mgm. in the injected animals, 212 in the control group.

It appears, therefore, that the effect of testosterone on the adrenals of the hypophysectomized-gonadectomized male rat is not secondary to its action on the testis or on the pituitary.

SUMMARY

Testosterone propionate causes partial repair of the atrophied adrenal cortex of hypophysectomized 26-28-day old female rats after post-operative intervals of 14 days, when injected for 10 days with a daily dose of 2.5 mgm.

Testosterone propionate partially maintained the adrenal weight and cortical lipid distribution in male rats hypophysectomized at 40 days of age and injected from the day of operation for 15 days with a daily dose of 2.5 mgm.

Testosterone propionate has a similar action on adrenal maintenance in the hypophysectomized rat which has been simultaneously gonadectomized. Therefore, the effect of the male sex hormone on the adrenals does not seem to be mediated through its action on the pituitary or the gonads.

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MECHANISMS OF DESOXYCORTICOSTERONE ACTION: EFFECTS OF THE WATER-SOLUBLE GLYCOSIDE ON HUMAN CIRCULATORY AND RENAL FUNCTIONS

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THE actions of the salt-active corticosteroids include a number of apparent inconsistencies. Contradictory effects have been reported in relation to water excretion, sodium excretion and glomerular filtration rate.

Desoxycorticosterone acetate (DCA) may be considered as representative of the salt-active corticosteroids. DCA injection increases both the diuretic response to water administration (Selye and Bassett, 1940) and the net daily water exchange (Green, 1948), yet its prolonged administration is followed by water retention and edema (Ferrebee, Ragan, Atchley and Loeb, 1939). Insufficiency of the salt-active corticosteroids, as observed in the adrenalectomized animal or the patient with Addison's disease, is characterized by inability to increase urine output in response to water loading (Robinson, Power and Kepler, 1941). Nevertheless, chronic water output is relatively excessive and the tendency to dehydration is marked.

The influence of salt-active corticosteroids on sodium excretion is more consistent; the usual immediate effect of DCA is sodium retention (Thorn, Engel and Eisenberg, 1939). However, its use in patients with Cushing's syndrome is followed by increased sodium output (Soffer, Lesnick, Sorkin, Sobotka and Jacobs, 1944).

The effect of the salt-active corticosteroids on glomerular filtration rate has also been the subject of conflicting data. DCA injection increases both the low filtration rate of the Addisonian patient (Talbott, Pecora, Melville and Consolazio, 1942) and the normal filtration rate of the dog (Collings, Downing and Hodges, 1949). Continued DCA administration to normal rats produces a decrease in filtration rate by the end of six weeks (Friedman, Polley and Friedman, 1948).

These apparent inconsistencies may be accounted for in most part on the assumption that the effects of the salt-active corticosteroids are the resultant primarily of opposed actions on water and sodium excretion (Gaunt, Birnie and Eversole, 1949) and secondarily of compensatory

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reactions evoked in response to the direct effects. The objective of the studies reported below was to determine the relative influence of these factors on the net action of desoxycorticosterone in human subjects. The glycoside of desoxycorticosterone (DCG) was used since its water solubility permits large intravenous doses to be given and immediate effects produced (Herrold, Hays, Holmquist, Richards and Oppenheimer, 1948). It was hoped that these characteristics would make it possible to distinguish direct effects from compensatory reactions.

METHODS AND MATERIALS

The experimental group was comprised of thirteen human subjects. The group included six patients with hypertensive cardiovascular disease, three with diabetes, one with idiopathic lymphedema, one with hyperthyroidism, one with postural hypotension and one with early nephritis. None was in congestive failure.

All studies were conducted after a twelve hour period during which no food or drink was allowed. The subjects were premedicated with 3 mgm. per kgm. of sodium phenobarbital given intramuscularly. The glomerular filtration rate was determined by the mannitol technique (Goldring and Chasis, 1944) and the renal plasma flow by the para-aminohippurate procedure (Smith, Finkelstein, Aliminosa, Crawford and Graber, 1945). Plasma and urinary sodium concentrations were measured by the uranium acetate method (Hoffman and Osgood, 1938). Blood pressure was estimated with a sphygmomanometer. Cardiac output determinations were made by the direct Fick method (Cournand, Baldwin and Himmelstein, 1949) in six of the subjects.

Blood samples were obtained from the femoral artery or the antecubital vein. Urine was collected through an indwelling soft rubber catheter. The bladder was washed out with distilled water after each collection and emptied completely by blowing out with air. All chemical analyses were done in duplicate. Pre-injection samples were used for blank determinations.

Control circulatory and renal functional values were determined during two or more 20 minute clearance periods. DCG was then given intravenously at a constant rate of 20 mgm. per square meter per hour, and the various measurements repeated during three additional 20 minute periods.

The mechanism of DCG action on water and sodium excretion was studied in relation to changes in filtration and reabsorption rates. The rate of tubular water reabsorption was estimated by subtracting the urine excretory rate from the glomerular filtration rate.

The rate of tubular sodium reabsorption was calculated as the difference between the urinary sodium output and the sodium filtration rate. The latter value was obtained by multiplying the glomerular filtration rate by the plasma sodium concentration.

RESULTS

The outstanding effect of DCG on renal function was a rise in water excretion (Fig. 1). The increase was most marked during the first test period, in which it averaged 53 per cent over the control value. The water output subsequently declined despite continued DCG infusion, but remained significantly higher than the preinjection level.

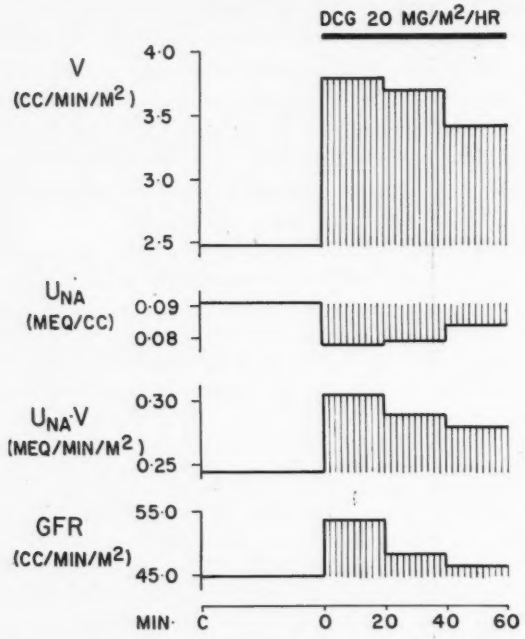

FIG. 1. Mean changes in renal water output (V), sodium concentration (U_{Na}), sodium output ($U_{Na} \cdot V$) and glomerular filtration rate (GFR) during continuous intravenous DCG injection.

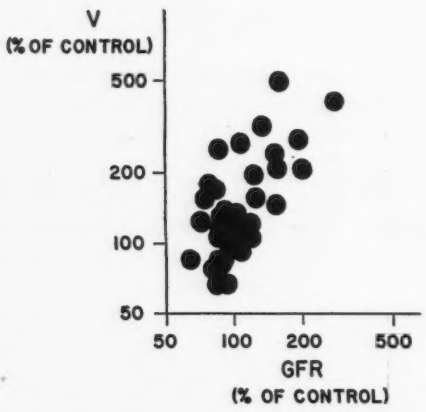

FIG. 2. Relation of percentage changes in water output (V) to percentage changes in glomerular filtration rate (GFR) during DCG injection.

The changes in water output were paralleled to some extent by alterations in glomerular filtration rate (Fig. 2). This association became less marked as DCG injection was continued. By the third test period the filtration rate had reverted to a value not significantly different from the control level, while the increase in mean rate of water excretion was maintained at three-quarters of the maximum reached during the first period. Calculation of reabsorption rates demonstrated that the tubular reabsorption of water had decreased below control levels in 11 of 37 determinations made during DCG injection, despite a rise in the rate of filtration.

The sodium output also manifested an early elevation, which correlated

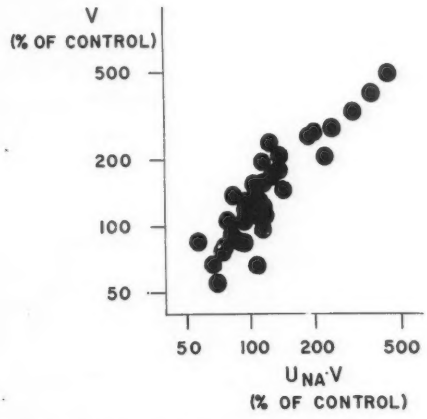

FIG. 3. Relation of percentage changes in sodium output ($U_{Na} \cdot V$) to percentage changes in water output (V) during DCG injection.

with the rise in water output (Fig. 3) and as a corollary, with increased filtration rate. The maximum increase in sodium excretion was less than half of that for water, and was followed by a more rapid decline. By the third test period, fifty per cent of the subjects had reduced their sodium excretion to values below the control level, despite the continued elevation of water output. The urinary sodium concentration fell an average of fourteen per cent immediately upon starting DCG injection and remained below the control level throughout all test periods (Fig. 1).

The mean circulatory actions of DCG were reviewed to determine whether the alterations in renal function were correlated with changes in blood pressure or flow (Fig. 4). No significant change in systemic blood pressure was noted during the period of administration. A small fall in cardiac output occurred during the first test period and was associated with an increase in peripheral resistance which averaged 20 per cent. Both

output and resistance subsequently reverted toward control values despite continued DCG administration. The change in peripheral resistance corresponded in magnitude and direction to the alteration in filtration rate. This relationship was interpreted to indicate altered efferent arteriolar resistance as a possible mechanism of the variations observed in the rate of glomerular filtration.

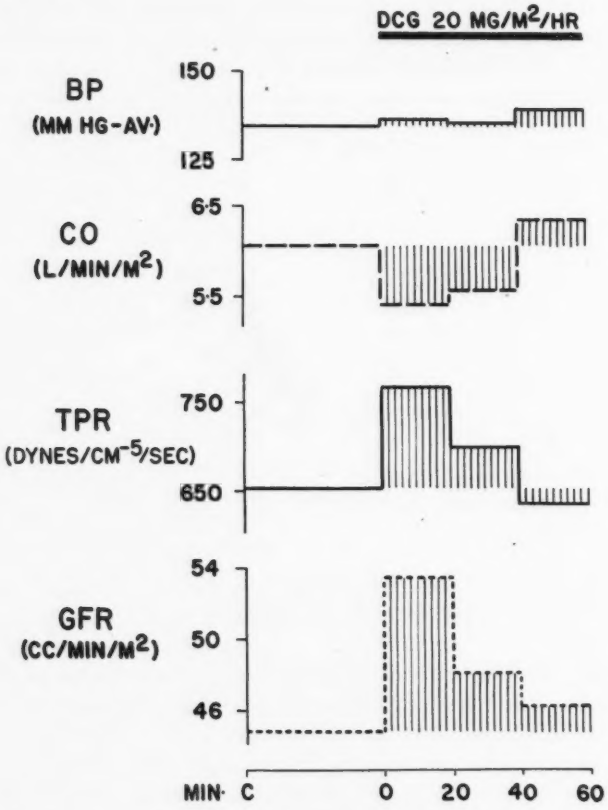

FIG. 4. Mean effects of continuous DCG injection on systemic blood pressure (BP), cardiac output (CO), total peripheral resistance (TPR) and glomerular filtration rate (GFR).

SUMMARY AND CONCLUSIONS

The effects of desoxycorticosterone glycoside (DCG) on circulatory and renal function were studied in 13 human subjects. The drug was given by continuous intravenous infusion at the rate of 20 mgm. per square meter per hour.

The renal action of DCG appeared to involve at least three effects: One effect consisted of an early, transitory rise in filtration rate, associated with a small increase in peripheral resistance and fall in cardiac output. Initial elevation of water and sodium output correlated in part with the rise in filtration.

A second effect was a reduction in the tubular reabsorption of water. This action was most evident in the elevated water output which persisted after reversion of the filtration rate toward control values.

The third effect was an alteration in sodium excretion. The net change appeared to be the resultant of two contrary forces: Increased water output and filtration rate were associated with a rise in sodium excretion. These factors were opposed by a relative increase in the tubular reabsorption of sodium, manifested early by a fall in urinary sodium concentration and later by reductions in total sodium excretion toward or below control levels despite continued elevation of water output.

Continuous DCG injection was not associated with significant change in systemic blood pressure.

ACKNOWLEDGMENT

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UTILIZATION OF RADIOACTIVE LIPIDES BY NORMAL AND HYPOPHYSECTOMIZED RATS[1]

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HYPOPHYSECTOMIZED rats were maintained in good condition for relatively long periods when purified rations containing either a relatively low or a moderately high amount of fat were fed *ad libitum* (Shaw and Greep, 1949). In the course of the above studies, it was observed that hypophysectomized rats fed the diet with the higher fat content appeared to be able to maintain themselves in somewhat sleeker condition than comparable animals fed the lower fat diet. However, the increases in body weight, the growth of the tail vertebrae, and the duration of survival were not affected by the fat content *per se*. Moreover, at autopsy, no appreciable difference in the total fat content of the carcasses of rats from the 2 diets was observed. Samuels, Reinecke and Bauman (1943) observed that hypophysectomized rats fed a semi-purified diet by stomach tube had a sufficiently high rate of fat retention as to suggest that, under the conditions of their experiments, hypophysectomized rats were not able to utilize fats at a normal rate. The contrast between these 2 series of experiments suggested the need to determine the respective ability of the hypophysectomized rat and the normal rat to utilize lipides.

EXPERIMENTAL

I. Studies with radioactive lipides

The ability of the hypophysectomized rat and the normal rat to use fatty acids was investigated with radioactive trilaurin and sodium octanoate. Both of these lipides were labeled in the carboxyl group with radiocarbon ($-C^{14}OO-$). Their preparation and properties have been described (Geyer, Matthews and Stare, 1949). The trilaurin had an activity of 920 counts per minute (c.p.m.) per mg. and was administered orally by intubation in the form of a finely dispersed emulsion which contained coconut oil (Geyer, Chipman and Stare, 1948; Geyer, Matthews and Stare, 1949). The octanoic acid had an activity of 6500 c.p.m./mg., but was diluted for

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use with varying amounts of an octanoate solution of very low activity (348 c.p.m./mg.). The actual total milligrams of octanoate and total c.p.m. used in each experiment are given at the bottom of the proper table. The octanoic acid, as its sodium salt, was injected intraperitoneally in aqueous solution. After administration of either of these compounds, the animal was placed immediately in a respirometer chamber through which CO_2-free air was drawn, and the respired CO_2 was collected in 1 N NaOH.

TABLE 1. UTILIZATION OF OCTANOATE BY THE NORMAL AND HYPOPHYSECTOMIZED RAT

Rat No.	Condition	Time interval	Respired CO_2	Activity in respired CO_2	Specific* activity	Injected C^{14} activity respired	Total injected C^{14} activity respired
		min.	mM	c.p.m.	c.p.m.	per cent	per cent
1†	Hypophysectomized, 210 days	0–60	3.71	18,090	406	46.4	46.4
		61–180	8.37	16,400	163	42.1	88.5
		181–300	8.16	2,990	30.5	7.7	96.2
	Total	300	20.24	37,480	—	96.2	—
2‡	Hypophysectomized, 12 days	0–60	7.09	20,000	236	56.8	56.8
		61–120	8.07	7,410	79	21.1	77.9
		121–180	8.03	2,310	24	6.6	84.5
		181–270	12.99	2,260	14	6.4	90.9
	Total	270	36.18	31,980	—	90.9	—
3§	Unoperated	0–60	8.52	23,850	234	63.1	63.1
		61–180	15.95	7,720	123	20.4	83.5
	Total	180	24.47	31,570	—	83.5	—

* Counts per minute per mg. of carbon.

† Rat No. 1 weighed 117 gm. and was injected with 29.2 mg. sodium octanoate with a total activity of 38,960 c.p.m.

‡ Rat No. 2 weighed 120 gm. and was injected with 33.5 mg. sodium octanoate with a total activity of 35,200 c.p.m.

§ Rat No. 3 weighed 120 gm. and was injected with 33.7 mg. sodium octanoate with a total activity of 37,800 c.p.m.

The alkaline solution was made up to volume and its CO_2 content determined in the Van Slyke and Neill apparatus. A suitable aliquot was treated with 0.5 N $BaCl_2$; the resulting $BaCO_3$ was washed and plated as described by Solomon, Gould and Anfinsen (1949). The dosage of the lipides, the times of CO_2 collection, the amount and activity of the respired CO_2, and other pertinent data are included in the table for each experiment.

The hypophysectomized rats used in these studies were males selected from larger groups of operated animals at periods varying from 12 to 210 days after surgery. In all cases, complete healing had occurred. Only those rats were selected which had no evidence of residual pituitary activity as demonstrated by lack of testicular maintenance.

At the end of the series of experiments, the hypophysectomized rats

were sacrificed, their heads fixed and serial sections made through the region of the sella turcica. Histologic examination confirmed the gross observations indicative of complete removal of the hypophysis.

Octanoate Utilization by Normal and Hypophysectomized Rats: The animals used had been maintained on a liver-containing synthetic-type ration (ration 100), the composition of which has been described (Shaw and Greep, 1949). This diet is one of those which has been shown to be capable of maintaining the hypophysectomized rat for long periods (Shaw and

TABLE 2. EFFECT OF DESICCATED THYROID OCTANOATE UTILIZATION BY THE HYPOPHYSECTOMIZED RAT

Rat No.	Diet	Time interval	Respired CO_2	Activity in respired CO_2	Specific activity	Injected C^{14} activity respired	Total injected C^{14} activity respired
		min.	mM	c.p.m.	c.p.m.	per cent	per cent
22*	Basal	0–60	3.15	14,020	327	39.2	39.2
		61–124	4.11	10,650	216	29.7	68.9
		125–197	4.16	3,330	66.6	9.2	78.1
		198–274	5.69	1,790	25.2	5.0	83.1
	Total	274	17.11	29,790	—	83.1	—
22†	Basal +1% dessicated thyroid	0–60	8.57	29,800	290	83.4	83.4
		61–129	9.81	7,000	83.4	19.6	103.0
		130–195	8.59	neg.	—	—	—
		196–288	12.57	neg.	—	—	—
	Total	288	39.54	36,800	—	103.0	—

* Body weight was 104 gm. Injected with 34.3 mg. of sodium octanoate with a total activity of 35,800 c.p.m.

† Body weight was 108 gm. Injected with 34.3 mg. of sodium octanoate with a total activity of 35,800 c.p.m.

Greep, 1949). The animals studied were: 1) two normal controls, 2) two recently hypophysectomized animals, and 3) four animals with long-standing hypophysectomy. Approximately 25 mg. of radioactive sodium octanoate were injected intraperitoneally per 100 gm. body weight. Typical results are given in Table 1.

Effect of Desiccated Thyroid on the Utilization of Octanoic Acid by the Hypophysectomized Rat: To determine whether raising the basal metabolism of hypophysectomized rats of long-standing affected the utilization of octanoic acid, 1 per cent of desiccated thyroid was incorporated into the diet of a suitable rat for 3 days prior to the experiment. During this period food consumption increased rapidly and the rate of respiration, as judged by CO_2 production, more than doubled. Sodium octanoate was given as before, and the results of this experiment are given in Table 2.

Utilization of Orally Administered Trilaurin by Normal and Hypophysectomized Rats: Emulsified radioactive trilaurin was given orally by intuba-

tion to normal and hypophysectomized rats at a level of approximately 1.0 ml. of emulsion per 100 gm. body weight. Each milliliter contained 25 mg. trilaurin and 25 mg. coconut oil. The results of the experiment in which the ability of a normal rat and an hypophysectomized rat to utilize trilaurin was studied are given in Table 3.

TABLE 3. UTILIZATION OF ORALLY ADMINISTERED RADIOACTIVE TRILAURIN BY HYPOPHYSECTOMIZED AND NORMAL RATS

Rat No.	Condition	Interval	Respired CO_2	Activity in respired CO_2	Specific activity	Injected C^{14} activity respired	Total injected C^{14} activity respired
		min.	mM	c.p.m.	c.p.m.	per cent	per cent
1*	Hypophysectomized	0–46	2.68	1,140	35.4	1.7	1.7
		47–120	4.03	4,850	100	7.2	8.9
		121–210	4.36	8,520	163	12.6	21.5
		210–279	2.94	7,360	208	10.9	32.4
		280–380	4.62	11,300	204	16.7	49.1
		381–515	7.06	12,150	144	18.1	67.2
	Total	515	25.69	45,320	—	67.2	—
2†	Unoperated control	0–60	8.89	2,650	24.9	3.4	3.4
		67–127	7.75	7,750	83.5	9.6	13.0
		128–220	11.59	18,200	131	22.5	35.5
		221–340	11.89	15,900	111	19.6	55.1
		341–375	4.23	2,350	46.3	2.9	58.0
	Total	375	44.35	46,850	—	58.0	—

* Rat 1 weighed 98 gm. and was intubated with 1.0 ml. of emulsion which contained 50 mg. trilaurin with a total activity of 67,500 c.p.m.

† Rat No. 2 weighed 124 gm. and was intubated with 1.2 ml. of emulsion which contained 60 mg. trilaurin with a total activity of 81,000 c.p.m.

II. A study on duration of survival of hypophysectomized rats

Immediately after complete hypophysectomy, 12 young, male, albino rats were distributed into two equal groups, of which one was fed ration100, with both liver concentrates omitted (ration 105) and the other the liver-free ration with one-fifth of the sucrose replaced by casein and the remainder by an isocaloric amount of lard (ration 165). Simultaneously, groups of 6 intact rats were maintained on each of these diets as controls. The rats on the low-fat, high-carbohydrate ration 105 survived on the average a little longer (17.0 days) than did the 6 rats on the high-fat, carbohydrate-free ration 165 (13.5 days), but the difference was not significant. There was no growth in any of these rats. However, control rats in both groups grew at normal rates.

DISCUSSION

Hypophysectomy does not interfere with the ability of the rat to utilize lipides as judged by the experiments reported in this paper. Both normal

and recently hypophysectomized rats used 60 per cent or more of a test dose of about 30 mg. of octanoic acid in the first hour after intraperitoneal injection. Approximately equal amounts of carbon dioxide were respired by animals in each of the above groups, presumedly because there was still an appreciable storage of thyroid hormone even though atrophy of the thyroid had begun in the operated animals. In animals of long-standing hypophysectomy, the absolute rate of octanoate utilization was lower than in comparable controls, since only 40–50 per cent of the octanoate was used in the first hour. However, such animals had a greatly lowered basal metabolism and respired about one-half of the normal quantity of carbon dioxide. When this lower overall metabolism was considered, the hypophysectomized rat was seen to be capable of oxidizing octanoic acid at a rate equal to or greater than the normal rat.

Desiccated thyroid ingestion increased the basal metabolism of the hypophysectomized rat and at the same time increased the absolute rate of octanoic acid utilization. Based on the amount of CO_2 produced, however, the specific rate of octanoic acid utilization was not increased. This fact supports the results discussed above, in that the level of basal metabolism and the rate of utilization of octanoic acid did not bear a linear relationship. The fact cannot be overlooked, of course, that the hypophysectomized rat consumed little food after atrophy of the thyroid had progressed and, thus, the supply of substrates with which the administered acid must compete might not be as great as in the normal animal. The significance of this potentially "competitive" effect is unknown at present.

Orally administered trilaurin was also well utilized by the hypophysectomized rat. This indicated that in pituitary-less animals absorption of fat from the intestine occurred readily and that the absorbed fat was oxidized at a normal rate. It is probable that the limiting step in the utilization of the trilaurin given orally was that of absorption, for it has been shown that intravenously administered trilaurin was oxidized much more rapidly than the present experiments indicated (Geyer, Chipman and Stare, 1948). Thus, somewhat greater differences in absolute rate of trilaurin utilization might result between normal and hypophysectomized rats as were found with octanoic acid, if the supply of substrate (trilaurin) had not been limited by the rate of absorption from the intestinal tract.

On the basis of the present observations, the high rate of fat retention observed by Samuels *et al.* (1943) in hypophysectomized rats would appear to be due to the introduction of an amount of food into the stomach in excess of what was required as a source of energy. This surplus would then result in an appreciable fat deposition.

The normal and hypophysectomized rats used in the tracer experiments had been maintained on a purified ration which contained 4 per cent liver

concentrates. In unpublished experiments conducted in this laboratory, there have been suggestions that these liver concentrates were necessary for the prolonged survival of the hypophysectomized rat. On the basis of present data, it does not appear possible to predict whether or not nutrients obtained from the liver concentrates were responsible for the pronounced ability of the hypophysectomized rat to utilize octanoic acid and trilaurin.

The short survival of the hypophysectomized rats used in this study to evaluate the ability to survive on low-fat and high-fat diets may have been due to the lack of liver concentrates in the rations. However, the appearance of the rats and the comparability of the average survival time for the 2 groups suggested that they were able to do equally well on the 2 diets.

SUMMARY AND CONCLUSIONS

The ability of normal and hypophysectomized rats to utilize octanoic acid and trilaurin labeled with radioactive carbon has been studied.

Recently hypophysectomized rats and normal rats had similar metabolic rates and used intraperitoneally administered octanoic acid at an extremely rapid and approximately equal rate. Rats with long-standing hypophysectomy had an extremely low rate of CO_2 production, low absolute rate of octanoic acid utilization but high utilization of octanoic acid in a relative comparison based on CO_2 production.

The ingestion of 1 per cent desiccated thyroid in the diet of rats with long-standing hypophysectomy resulted in a great increase in CO_2 production, increased absolute rate of octanoic acid utilization but no increase in relative utilization of octanoic acid based on CO_2 production.

Hypophysectomy of long duration in rats reduced the absolute rate of trilaurin utilization slightly but allowed a slightly increased utilization above normal in terms of relative CO_2 production.

Hypophysectomized rats maintained themselves approximately as well on a diet with low-fat, high-carbohydrate levels as on a diet deficient in carbohydrate but with a high-fat level.

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SOME EFFECTS OF PURIFIED PITUITARY GROWTH HORMONE ON CARBOHYDRATE METABOLISM IN THE RAT[1]

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CRUDE pituitary extracts were shown in early work to prevent the hypoglycemic action of insulin, to produce hyperglycemia and glycosuria in dogs and cats when given in excess, to increase glycogen storage and lower the respiratory quotient in rats, and to promote ketogenesis and mobilization of fat to the liver in fasting animals. Later experiments indicated that a significant proportion of these effects on carbohydrate metabolism might be mediated through the action of the adrenocortical hormones. However, adrenalectomy did not eliminate the effects of pituitary extracts on muscle glycogen maintenance in hypophysectomized rats (Russell and Bennett, 1936; Bennett and Perkins, 1945), nor the ability of these extracts to antagonize the action of insulin in rabbits (Himsworth and Scott, 1938), nor the ketogenic activity of such extracts in rats (Shipley, 1940). Similarly, diabetogenic effects were seen in partially depancreatized dogs from which the adrenals had been removed (Houssay *et al.*, 1946). It may be noted that in most of this work the hormone preparations used were saline or alkaline extracts of bovine pituitaries; these, it is now known, are usually poor in adrenocorticotrophin, but rich in growth hormone. Thus, it appeared probable that whole pituitary extracts contained factors in addition to the adrenotrophic hormone which could affect carbohydrate metabolism, and that one of these substances might conceivably be the growth hormone.

More recent work with concentrated or purified growth hormone has

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confirmed the impression that this hormone may affect carbohydrate and fat metabolism as well as nitrogen storage. Young (1944) found that pituitary extracts which induced growth in puppies were diabetogenic in older dogs. Marx *et al.* (1943), Bennett and Li (1947), and Gaarenstrom *et al.* (1949), employing partially purified or pure growth hormone preparations, observed an increase in the glycosuria of paritally depancreatized or alloxandiabetic rats. Growth hormone and adrenotrophin both were shown to enhance ketone body formation (Bennett *et al.*, 1948) and to increase the liver fat content (Li *et al.*, 1949) in fasting normal rats. Szego and White (1949) also reported the accumulation of liver fat in fasted normal and adrenalectomized mice given growth hormone. Quite recently the production of a temporary diabetic state in normal cats and dogs with purified growth hormone has been reported by Cotes, Reid and Young (1949), Houssay and Anderson (1949), and Campbell *et al.* (1950).

The experiments to be reported here were designed to determine whether or not some of the other effects of pituitary extracts, referred to above, might be attributable to the growth hormone.

METHODS AND MATERIALS

The experimental animals used were young adult male rats of the Sprague-Dawley strain, 250 to 350 gm. in weight. Diabetes was induced by the subcutaneous injection of alloxan (17 mg. per 100 gm. in 2 per cent solution), usually after a fasting period of 48 hours. In most instances, the diabetic animals were used for experiment between 2 and 5 weeks after treatment with alloxan. Typical blood glucose levels seen in these animals in the fed and fasted states are included in Table 3. Those animals which were adrenalectomized were maintained usually with desoxycorticosterone acetate (subcutaneous pellet), or in some cases with saline drinking fluid; the completeness of adrenalectomy was established by death of the animals within 1 week following the removal of the pellets or of the salt. Unless otherwise stated, the animals were fasted for 18 to 24 hours before each experiment.

The growth hormone preparations used were made by the method of Wilhelmi, Fishman and Russell (1948) . The purified hormone was crystalline and electrophoretically homogeneous. In some experiments a partially purified preparation was used. This was in most instances a "B" fraction (precipitated from the crude alkaline extract of beef pituitaries at pH 7 to 7.5 with ethyl alcohol concentrations between 12 and 24 per cent) which contained about 50 per cent growth hormone as judged by electrophoretic analysis. The results obtained with this preparation were identical with those yielded by the purified hormone. Neither the purified nor semi-purified materials contained detectable adrenotrophic hormone, as judged by the bioassay method of Sayers *et al.* (1947). The hormone samples were preserved as lyophilized powders and dissolved immediately before use in physiological saline (made slightly alkaline if necessary to effect solution). They were administered intraperitoneally in a volume of about 0.5 ml.

Blood samples (0.1 to 0.3 ml.) were obtained from the tail without anesthesia. Blood glucose determinations were made photometrically on copper tungstate filtrates (Somogyi, 1941) by means of the phosphate-buffered copper reagent of Somogyi (1945) and the arsenomolybdate reagent of Nelson (1944). Amino nitrogen was estimated photo-

metrically (Russell, 1944) on tungstic acid filtrates of whole blood. Muscle glycogen was estimated by a modification of the method of Good, Kramer and Somogyi (1933), the samples being taken under pentobarbital anesthesia. Respiratory measurements were made with an open-circuit Haldane apparatus during 2-hour periods.

EXPERIMENTAL DESIGN AND RESULTS

1. *The acute effects of growth hormone on the blood glucose of fasted rats:* Both hypoglycemia and hyperglycemia have been reported to occur following the administration of crude anterior pituitary extracts to animals. Usually hypoglycemia has been seen as an acute effect in fasted animals. Hoffman and Anselmino (1933) and Weinstein (1939) reported the effect

TABLE 1. THE EFFECT OF GROWTH HORMONE ON THE BLOOD GLUCOSE OF NORMAL RATS

Experiment	No. of animals	Growth hormone given (mg. per 100 gm.)	Initial blood glucose* (mg. per cent)	Hours after growth hormone: 1	2	3	4	5	6	7–8
				Average blood glucose—mg. per cent						
1. Fasting animals given growth hormone										
A. Semipurified G.H.	5	5	81	70	57	56	60	63	54	52
B. Purified G.H.	5	3	78	65	62	53	54	61	40	54
				Change from initial level—mg. per cent						
Combined series (av.):			79	−11	−20	−25	−23	−18	−29	−26
Combined stand. error:			±1.4	±2.0	±3.0	±2.2	±1.8	±1.5	±2.8	±2.7
Combined no. observations:			(10)	(8)	(10)	(8)	(9)	(9)	(5)	(6)
				Change from initial level—mg. per cent						
2. Fasting animals, control observations										
A. Untreated (bled hourly)	5	—	79 ±2.5		0 ±1.7		− 3 ±1.5		− 1 ±2.3	− 6‡ ±1.9
B. Inactivated hormone	3	3	68	− 3 ±0.3	+ 1 ±6.1	+ 2 ±2.9				
3. Unfasted animals given growth hormone	6	3	99 ±2.6	+ 2 ±4.1	− 3 ±1.5	−16‡ ±6.0				

* The standard deviation (within groups) of initial blood glucose levels: ±5.0 (24 obs.); of the changes in blood glucose: ±6.0 (89 obs.).
† 9 hours after initial observations.
‡ 3 observations at 3 or 4 hours.

in dogs, Harrison and Long (1940) in normal and adrenalectomized rats, and Horsters (1933), using a "thyrotrophic" preparation, in rabbits. Hyperglycemia or glycosuria develops only in fed animals, usually after several days of treatment (see Houssay, 1936). Hyperglycemia has not been seen in normal rats, but in partially depancreatized rats, it occurs readily, as evidenced by marked glycosuria developing within the first day following the injection of crude anterior pituitary extract (Long, 1947). One report of an effect of growth hormone on the blood glucose has appeared, that of Marx *et al.* (1944), who found that a partially purified preparation induced hypoglycemic convulsions in fasted hypophysectomized rats in 1 to 3 hours after its administration.

In the present series of experiments, the immediate responses of normal, adrenalectomized, and alloxan-diabetic rats to purified growth hormone were studied. After fasts of 18 to 24 hours, the animals were given the hormone in amounts averaging 3 mg. of the pure material per 100 gm. body

weight. Blood samples were then obtained at intervals, usually hourly, for 4 to 8 hours, and analyses made of the glucose or amino nitrogen concentrations. The amino nitrogen data will be presented below.

The effects of the hormone on the blood glucose of normal rats, and some control data, are recorded in Table 1. It can be seen that in the fasted animal with a normal pancreas, growth hormone induced an immediate fall in the blood sugar to hypoglycemic levels (45 to 55 mg. per cent) which were maintained for at least 8 hours. Physical signs of hypoglycemia were seen between the 2d and 3d hours. A small but apparently significant transitory rebound usually occurred between the 4th and 6th hours, but

TABLE 2. THE EFFECT OF GROWTH HORMONE ON THE BLOOD GLUCOSE OF FASTED ADRENALECTOMIZED OR ADRENALECTOMIZED-DIABETIC RATS

	No. of obs.	Growth hormone given* (mg. per 100 gm.)	Initial blood glucose (mg. per cent)	Hours after growth hormone: 1	2	3	4	5	6	8
				Blood glucose of individual animals—mg. per cent						
1. Adrenalectomized rats										
A. With sub-cut. DOC pellets		5 S	60	52	38	28†	40	56		
		5 S	80	42	25†	18‡				
		2.5 S	58	49	45	42		37	33†	56
		2.5 S	61	53	53	51	45	42	33†	56
				Average change from initial level						
	4		63	−16	−25	−30				
B. With saline drinking fluid	4	3 P	58 ±1.2	−19 ±1.9						
2. Adrenalectomized-diabetic rats	9	3–4 P	70 ±4.0	+ 3 ±1.7	+ 5 ±1.9		− 7 ±1.6			

* S, semipurified; P, purified growth hormone.
† Comatose; cortical extract given after this sample (1 ml. Lipoadrenal extract (Upjohn) except in the second animal which was given aqueous extract).
‡ Died.

the level then fell again and remained low. The response of normal rats fasted 4 days (not included in the table) was the same as that of those fasted 1 day. Neither the procedures of handling and bleeding the animals every hour, nor the injection of inactivated hormone (heated in boiling water for 30 minutes) had any effects on the blood glucose level.

In adrenalectomized rats, in which the fasting blood glucose was already low, the hypoglycemia following growth hormone often produced convulsions and death (Table 2). Several other animals, in addition to those recorded in the table, were lost in this way. The administration of adrenal cortical extract could bring about resuscitation of the animals, with elevation of the blood glucose. For reasons not immediately apparent, adrenalectomized rats which had been maintained with salt, rather than with desoxycorticosterone, were not so severely affected, although the blood sugar reached quite low levels. Adrenodemedullated rats (not shown) responded to growth hormone in a fashion similar to normal rats, except that no significant rebound was seen.

In animals with reduced pancreatic function, on the other hand, hypoglycemia was never induced; instead there was a prompt elevation of the blood glucose which reached a maximum in about 4 hours after the injection of the hormone (Table 3). It should be noted that the hyperglycemic response occurred in all the alloxan-treated animals (and a partially depancreatized rat), although the degree of severity of the diabetes varied

TABLE 3. EFFECT OF GROWTH HORMONE ON THE BLOOD GLUCOSE OF FASTING ALLOXAN-DIABETIC RATS

	No. of animals	Growth hormone given* (mg. per 100 gm.)	Fed blood glucose† (mg. per cent)	Initial blood glucose (fasting) (mg. per cent)	Hours after growth hormone						
					1	2	3	4	5	6	8
					Change in blood glucose—mg. per cent						
1. Diabetic rats											
A. Growth hormone treated		3 P	269	74	+47	+ 77	+104	+134			
		3 P	530	107	+60	+ 97	+152	+216			
		5 P	244	110			+ 32	+ 66	+102		
		5 S	274	118	+12	+ 39	+ 72	+ 92	+ 82	+63	+23
		3 P	417‡	135	+60	+ 75		+144			
		5 S	434	144		+ 23	+ 66				
		3 P	437	245		+ 15		+ 19		−36	
		4 P		269	+23	+ 81	+ 93	+ 99	+ 67	+37	+25
		3 P	750	286	+33	+ 54	+ 85	+ 90	+ 57		
		3 P	469	331		+ 27		+ 65		+65	
		3 P	648	361	+75	+117	+117	+123	+102		
average	11	—	447	198	+44	+ 61	+ 90	+105	+ 82	+32	
B. Inactivated hormone	4	—		268	− 8 ±19	− 15 ± 21	− 10 ± 13				
C. Serum albumen (human) 4 mg. per 100 gm.	8	—		264					− 37 ± 11		
D. Adrenocorticotrophic hormone (0.04 mg. per 100 gm.)	4	—		281	−33 ±15	− 18 ± 11	− 57 ± 40				

* P, highly purified hormone; S, semipurified hormone.
† Determined a few days before the experiment.
‡ Partially depancreatized at 1 mo. of age; experiment 14 weeks postoperative.

from mild to extreme. That is, those animals with residual insulin-secreting cells, as evidenced by the normal level of fasting blood sugar, but with impaired carbohydrate tolerance, responded to growth hormone similarly to those with very severe diabetes. When inactivated hormone or nonspecific protein was given to the diabetic animals, the blood glucose did not change or tended to fall.

The hyperglycemic responses of a few diabetic rats fasted 2 or 3 days (not shown in Table 3) were less than in those fasted 1 day only. Those in which hyperglycemia occurred were found to have abnormally high levels of liver glycogen; where fasting eliminated the hyperglycemic response, the liver glycogen was low. Similar high concentrations of glycogen in the livers of 24-hour fasted diabetic rats have been observed by others (Janes *et al.*, 1946; Tuerkischer and Wertheimer, 1946). It may be that adequate liver glycogen stores are required for the hyperglycemia to become manifest; but the mechanism of growth hormone action here cannot

be one only of glycogenolysis, since normal unfasted rats with high liver glycogen did not show an elevation of the blood glucose when given the hormone (Table 1).

Table 3 includes also data obtained from diabetic rats given a small amount of adrenotrophic hormone (ACTH). The blood glucose tended to fall here, rather than to rise. The amount of ACTH employed, 40 μg. per 100 gm., was equivalent to that which might have been contained in the growth hormone preparations used if these had been contaminated with ACTH to the extent of 1 per cent (above the maximum possible figure as indicated by bioassay). It is evident that ACTH in the growth fractions could not be responsible for the hyperglycemic activity. It is of course possible that larger amounts of ACTH would raise the blood glucose.

Since growth hormone produced hyperglycemia in diabetic rats and severe hypoglycemia in adrenalectomized animals, it was of interest to determine its effect on the blood glucose of fasted adrenalectomized-diabetic rats. These animals were adrenalectomized some weeks after the induction of diabetes and observed a week or more later. In Table 2 (section 2) it can be seen that such animals were not subject to severe hypoglycemia like adrenalectomized rats which were otherwise intact. On the other hand, the hyperglycemic response of diabetic rats was either greatly modified or abolished by adrenalectomy. This failure may have been the result of low liver glycogen stores, which could be expected in such animals (Janes *et al.*, 1946), or it may have been an expression of a necessary degree of synergism between the growth and adrenal hormones (Russell, 1940).

These observations on the effect of growth hormone on the blood glucose indicate that the initial response in the normal fasting rat is the induction of a hypoglycemic phase. Since this effect is not seen in rats with reduced pancreatic function (intact or adrenalectomized), it may be postulated to be the result of stimulation of the islet cells by direct or indirect means. The elevation in blood glucose seen even in mildly diabetic rats then may be akin to the "diabetogenic" effects of growth hormone observed in other species.

2. *The effect of growth hormone on the blood amino nitrogen in fasting normal and diabetic rats:* Diminution in the concentration of amino nitrogen in the blood, presumably concomitant with retention of nitrogen in the tissues, has been observed whenever pituitary preparations rich in growth hormone have been given. Purified growth hormone has been shown to have the same effect (Li, Geschwind and Evans, 1949). Insulin, too, lowers the blood amino nitrogen, and it has been suggested for this and other reasons that insulin is essential for the nitrogen-retaining action of growth hormone. Since the effects of growth hormone on the

blood glucose suggest increased secretion of insulin in normal rats, it was of interest to observe any accompanying changes in blood amino nitrogen in normal and diabetic animals. Data from such experiments are summarized in Table 4. With the doses of hormone used, the fall in blood amino nitrogen, observed in all the animals, bore no apparent relationship to the direction or degree of alteration in the blood glucose. If the diabetic animals were unable to increase their output of insulin in response

TABLE 4. EFFECT OF GROWTH HORMONE* ON THE BLOOD AMINO NITROGEN OF FASTING DIABETIC RATS

	No. of observ.	Initial blood amino N (mg. per cent)	Hours after growth hormone			
			2	4	5	6
			Change in blood amino—mg. per cent			
Series 1						
Normal rats	4	12.8 ±0.75	−3.0 ±0.6	−5.4‡ ±0.4		−4.1 ±1.0
Diabetic rats†	5	13.9 +0.53	−2.4‡ ±0.2	−4.1 ±0.7		
Series 2						
Normal rats	9	11.5 ±0.29			−2.7 ±0.39	
Diabetic rats§	9	12.3 ±0.20			−2.2 ±0.30	
Untreated diabetic rats‖	8	11.0 ±0.22			−0.2 ±0.23	

* Purified growth hormone, 3 mg. per 100 gm.

† Data on the blood glucose changes in most of these animals are recorded in Table 3. Of the animals included here, the average fed blood glucose was 590 mg. per cent, fasted blood glucose 250 mg. per cent, and the increase 4 hours after growth hormone +85 mg. per cent.

‡ Two observations only.

§ In this series, the average fed blood glucose level was 410 (310–509) mg. per cent, the fasted (initial) value 174 (105–304) mg. per cent, and the average increase in blood glucose 5 hours after growth hormone +50 ±14 mg. per cent (not included in Table 3).

‖ Given human serum albumin 3 mg./100 gm.

to growth hormone, as suggested by the effects on the blood glucose, then it appears that increased secretion of insulin is not responsible for the blood amino nitrogen-depressing effect of the growth hormone. Since the animals were probably not wholly lacking in insulin, however, it is not possible to say whether minimal quantities of insulin may not be needed.

3. *The effect of growth hormone on the action of insulin in fasted rats:* Since the original observations of Houssay, a number of investigators have confirmed in a variety of species the fact that prior treatment with anterior pituitary extracts can confer normal resistance to insulin on hypophysectomized animals or render intact animals abnormally refractory. The work of Jensen and Grattan (1940) and Grattan and Jensen (1940) indicated that adrenotrophic hormone and adrenal steroids,

through their glycogenic action, could protect normal mice from the convulsive action of insulin. Several other pituitary hormones were found ineffective, but growth hormone was not available in sufficiently purified form for testing at that time. In view of the contra-insulin activity of crude beef pituitary extracts, noted by several workers and the effects seen in adrenalectomized rabbits by Himsworth and Scott, it appeared that there could be more than one pituitary factor having contra-insulin activity. Accordingly, growth hormone has been tested in this respect.

TABLE 5. EFFECT OF GROWTH HORMONE ON THE INSULIN SENSITIVITY OF NORMAL FASTING RATS

	No. of obs.	Initial blood glucose (mg. per cent)	Hours after insulin			
			0.5	1.0	1.5	2.5
			Change in blood glucose—mg. per cent			
A. All data						
1. Insulin alone (0.5 U./kg.)	10	81 ± 3	−26 ± 3	−37 ± 3	−33 ± 4	−16 ± 5
2. Growth hormone* plus insulin	12	55 ± 3	− 9 ± 3	− 5 ± 3	+ 2 ± 2	
B. Selected data†						
1. Insulin	4	73 ± 1	−24 ± 2	−31 ± 4	−27 ± 5	−13 ± 6
2. Growth hormone plus insulin	4	67 ± 1	− 4 ± 2	− 4 ± 4	− 2 ± 3	

* Growth hormone in doses of 2 to 5 mg. per 100 gm. was given 2 to 6 (usually 4) hours before insulin. Of the 12 animals, 5 were given purified hormone, 7 the semipurified "B" fraction; there was no difference between the average responses to the two types of preparation.

† From the group given insulin alone, animals with the lowest initial blood glucose levels; from the group given growth hormone and insulin, those with the highest initial values.

The effect of pretreatment with growth hormone on the response to insulin was examined in fasting normal and adrenalectomized rats. The observations in normal animals are presented in Table 5. Preliminary observations with crude extracts had indicated the maximum effects to be obtained when the hormone was given between 3 and 12 hours before the insulin; in the experiments reported, the usual interval between treatments was 4 hours. The first section of Table 5 shows that the hypoglycemic action of insulin in normal rats was greatly modified by previous treatment with growth hormone. The fall in blood glucose was small, and in most of the animals the original level was regained in 1 hour; in the control groups, the blood sugar was still depressed after 2½ hours.

The interpretation of this effect is complicated by the fact that the initial blood glucose levels in the animals given the growth hormone were much lower than in the untreated rats. It might be considered that the magnitude of the response to insulin would be expected to be less if the blood glucose

level at the time of its injection were low. A partial answer to this objection is presented in section B of Table 5, where the responses of the 4 control animals with the lowest fasting blood sugar values are compared with those of the treated group which had the highest initial levels. The changes in blood glucose induced by insulin in the selected groups are not different from the average changes in the groups from which the selections were made. The small difference between the initial blood glucose levels in the selected groups does not seem sufficient in itself to account for the apparent degree of resistance to insulin in the treated animals.

There is still the possibility that the contra-insulin action of the growth hormone in these experimental conditions was brought about in an indirect fashion. Somogyi has suggested that even quite small degrees of hypoglycemia may, in intact individuals, set in play reactive mechanisms. We have observed in a few experiments that the prior administration of protamine insulin resulted in a diminished response to the test dose of regular insulin. Further, in some of the normal animals in which hypoglycemia was induced by the growth hormone, the adrenal ascorbic acid was found to be diminished by about 40 per cent, indicating secretion of adrenotrophic hormone in these conditions (see Gershberg and Long, 1949). It is evident, however, that the amounts of cortical hormone which may have been released were insufficient to overcome the hypoglycemic action of the growth hormone; presumably also they would be insufficient to obscure the action of exogenous insulin.

If the growth hormone is a contra-insulin agent per se, its action might be expected to be demonstrable in the adrenalectomized rat. Our attempts to carry out this experiment have been unsatisfactory because fasting adrenalectomized rats have lower fasting blood sugar concentrations than normal animals. The effect of the growth hormone here was to reduce the blood glucose to precarious levels, so that the later injection of insulin produced convulsions in many animals. In a small group of rats which remained in relatively good condition, the tendency of the growth hormone treatment was to reduce the effect of insulin on the blood glucose. It is possible that more definite contra-insulin activity might be seen in adrenalectomized animals subjected to a shorter fast or given smaller doses of insulin.

4. *The effects of growth hormone on the utilization of feed carbohydrate in the normal rat:* When glucose is fed to normal fasting rats, the prior administration of crude anterior pituitary extracts diminishes the respiratory quotient and increases the deposition of glycogen in muscle (Myer, Wade and Cori, 1937; Russell, 1938). The interpretation of these effects in terms of diminution of carbohydrate utilization was further supported by the observations made in eviscerated rats, where the pituitary extract di-

minished the rate of removal of blood glucose by the peripheral tissues (Russell, 1943). Harrison and Harrison (1942) have observed also elevated glucose tolerance curves in rats pretreated for 24 hours with similar anterior pituitary extracts. The depression of the R.Q. of carbohydrate-fed rats was utilized by Greaves *et al.* (1940) as an assay method in the course of fractionating bovine pituitary extracts; in this work, the factor affecting the R.Q. could not be separated from that having growth-promoting activity. Table 6 presents data obtained from similar experiments made

TABLE 6. EFFECT OF GROWTH HORMONE ON THE RESPIRATORY QUOTIENT AND MUSCLE GLYCOGEN DEPOSITION OF CARBOHYDRATE-FED NORMAL RATS

	No. of animals	Respiratory quotient	Muscle glycogen (per cent)
Untreated rats	6	0.846 ± 0.005†	0.759 ± 0.008
Growth hormone* treated rats	13	0.759 ± 0.010	0.836 ± 0.009

* Growth hormone (3 mg. per 100 gm.) was given 2 hours before glucose was fed. Respiratory measurements were made over 2-hour periods following the glucose feeding, and the glycogen analyses made at 3 hours. Of the 13 treated rats, 6 were given purified hormone and 7 semipurified material; there was no difference between the mean responses thus obtained.
† Standard error.

TABLE 7. EFFECT OF GROWTH HORMONE ON THE GLUCOSE TOLERANCE OF NORMAL FASTED RATS

	No. of animals	Initial blood glucose (mg. per cent)	Hours after glucose		
			0.5	1.0	1.5
			Change in blood glucose—mg. per cent		
Untreated rats	6	71 ± 2.7†	+57 ± 2.3	+40 ± 8.3	+23 ± 2.7
Growth hormone* treated rats	8	54 ± 4.3	+99 ± 7.3		+41 ± 4.7

* Purified growth hormone, 3 mg. per 100 gm., 4 hours before glucose.
† Standard error.

with purified growth hormone. Normal fasted rats (weighing 250 ± 10 gm.) were force-fed 5 ml. of 20 per cent glucose solution 4 hours after the intra-peritoneal injection of growth hormone or of saline. Respiratory measurements were then made, and 3 hours after the glucose was fed, the gastrocnemii muscles were taken for glycogen analysis. The glycogen values are expressed as gm. of glucose per 100 gm. of fresh tissue. It is apparent that purified growth hormone, like crude pituitary extracts, depresses the R.Q. and increases the glycogen content of muscle in glucose-fed animals.

In another series of experiments the effect of growth hormone on the glucose tolerance curve was examined. Isotonic glucose, 100 mg. per 100

gm., was given intraperitoneally to fasting rats which had been injected with growth hormone or saline 4 hours previously. The blood sugar changes are summarized in Table 7. The ability of the rat to remove glucose from the blood appeared to be somewhat but not markedly diminished by such treatment. As in the insulin sensitivity test, the initial hypoglycemia following growth hormone makes the significance of the observations somewhat uncertain. However, as in that case, comparison of those of the treated animals having the highest blood sugar values (av. 64 mg. per cent) with those of the controls with the lowest levels (av. 66 mg. per cent) showed the same difference in tolerance to persist. Since the normal rat treated acutely with growth hormone takes up glucose rapidly to form muscle glycogen, it is not surprising that the effects on the blood glucose are small.

DISCUSSION

From these and other observations it appears that many of the effects of crude anterior pituitary extracts on carbohydrate metabolism can be attributed to the growth hormone. Since nothing is known of the intimate relationship in the tissues between the formation and retention of protein and the metabolism of carbohydrate and fat, it is not yet possible to formulate a mechanism for the action of the growth hormone jointly on these two phases of metabolism. Logically it may be presumed that a single point of action common to the several processes would be affected by the hormone.

An immediate and prolonged reduction of the fasting blood sugar level was the most obvious effect of a single injection of growth hormone on normal rats. The fact that this was seen only in animals with an intact pancreas and not in those with reduced islet function suggests that the hypoglycemia is the result of increased insulin secretion. This interpretation is in accord with the observations of several investigators that pituitary extracts may elicit proliferation of the islet tissue in the rat (see Richardson and Young, 1937), the dog (Richardson and Young, 1938; Ham and Haist, 1941), and the rabbit (Oglivie, 1944), and an increase in the insulin content of the rat pancreas (Marks and Young, 1940; Funk, *et al.* 1941; Fraenkel-Conrat, *et al.*,[3] 1942). Young (1944) has presented evidence suggesting that the secretion of insulin may be increased during the early phases of chronic pituitary treatment in the dog. Here the insulin content of the pancreas is found reduced (Best, Campbell, and Haist,

[3] These investigators also reported a decrease in insulin content of the pancreas following treatment with a partially purified growth hormone preparation and with adrenocortical extract, an increase with adrenotrophic fractions, and variable effects of lactogenic hormone. These data require confirmation with the purified hormones now available.

1939) and later the α cells of the islets severely damaged (Best *et al.*, 1942). Some observations of Conn, *et al.* (1945) suggest also a "pancreatrophic" effect in human subjects; two patients with presumed hyperinsulinism (one having an islet cell tumor) were treated with a crude pituitary extract which was diabetogenic in dogs, with the result that the post-absorptive blood glucose levels of the patients were considerably diminished during the course of treatment. It has been suggested by Lukens (1946) and others that the islets first are stimulated by hyperglycemia induced by the pituitary factor and later become exhausted by the continued strain placed upon them. In the present experiments in the rat, it is evident that hypoglycemia occurred prior to any elevation in the blood sugar, so that if an increased secretion of insulin took place, it must have been initiated by other mechanisms. The nature of the stimulus to the islets is problematical. Anderson and Long (1947) observed in the isolated perfused rat pancreas a decrease rather than an increase in the release of insulin when growth hormone was added to the perfusate. If this is true, increased activity of the islets must be induced indirectly, perhaps through increased utilization of insulin during the period of growth hormone action, or through changes in blood constituents other than glucose.

The hyperglycemic action of the growth hormone seen in rats with any degree of reduction in insulin-secreting tissue may be similar to the diabetogenic effects of the hormone observed in adult dogs and cats. The course of the metabolic changes produced in the latter animals by pituitary extract can be reproduced in rats in which the amount of pancreas has been earlier reduced by operation or by alloxan, and young dogs too may be made more susceptible to pituitary diabetes by prior partial pancreatectomy. We have been able to increase considerably the glycosuria of mildly diabetic rats by chronic treatment with growth hormone (unpublished). Variation in the responses of different types of animals to pituitary extract and to growth hormone might be in part explained by supposing that adult dogs and cats have less reserve of pancreatic function than young dogs or than rats, and that the full effects of such diabetogenic substances become more easily demonstrable when increased secretion of insulin takes place with difficulty. Such a suggestion has been made also by Young (1945).

The mechanism by which the hyperglycemia was exaggerated in the diabetic rats is not yet known. It now appears that the growth hormone is the factor affecting the R.Q. and muscle glycogen deposition in carbohydrate-fed animals and the maintenance of muscle glycogen in fasting hypophysectomized rats (Russell and Wilhelmi, 1950); and it may also be in part responsible for the anti-insulin activity of anterior pituitary extracts. The growth hormone seems then to have the effect of diminishing

the net utilization of carbohydrate, and this action would be expected to play a part in raising the blood glucose. As noted previously (Russell, 1940) this type of activity may not be wholly independent of adrenocortical function, but requires for some part of its effects the presence of synergistic amounts of cortical hormone.

Depression of the blood amino acids in response to growth hormone occurred to a similar extent in rats with all degrees of pancreatic function, in contrast to the changes in the blood sugar induced by the hormone, so that an *increase* in insulin secretion would not appear to be required for the effect of growth hormone on blood amino nitrogen. However, a quantitative relationship between diminution in circulating amino acids and retention of nitrogen in the tissues in response to the hormone has not been established. The present observations therefore do not provide evidence against the view that insulin may be required in normal or increased amounts for maximal or prolonged retention of nitrogen.

SUMMARY

Purified pituitary growth hormone was given in acute experiments to adult rats. In normal fasting animals a single injection of 3 mg. per 100 gm. was followed by moderate hypolgycemia lasting at least 8 hours. In adrenalectomized rats the hypoglycemia was severe and sometimes fatal. Alloxan-diabetic rats treated similarly responded with elevation of the blood glucose, rather than diminution, the increase reaching a variable maximum in about 4 hours. This effect was seen in animals with diabetes of all grades of severity. In adrenalectomized-diabetic rats, growth hormone had no marked effects on the blood glucose. The hypoglycemic phase was therefore suggested to be the effect of an increase in the secretion of insulin, and the hyperglycemic reaction seen when pancreatic function was diminished to be the result of a more immediate action of the hormone on the metabolism of carbohydrate. The blood amino nitrogen was decreased by growth hormone to a similar extent in normal rats and in those with any degree of diabetes.

Growth hormone given to normal fasting rats 2 to 4 hours before experiment had also the following actions: the further hypoglycemic effect of a test dose of insulin was largely prevented; some elevation in the peak of the glucose tolerance curve was seen; and in rats fed glucose, the R. Q. was depressed and deposition of muscle glycogen increased as compared to the values seen in untreated animals similarly fed.

ACKNOWLEDGMENT

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NOTES AND COMMENTS

ESTRUS AND MATING IN GONADECTOMIZED FEMALE MICE WITH ADRENAL CORTICAL ABNORMALITIES[1]

Previous papers have described the effects of early gonadectomy on *DBA* and *CE* strain mice, and on the F_1 offspring of reciprocal crosses between these two strains. *DBA* females develop adrenal cortical hyperplasia within 2–7 months after ovariectomy (Fekete *et al.*, 1941), *CE* females develop adrenal cortical carcinoma within 6–12 months (Woolley and Little, 1945a), and the hybrids also show adrenal cortical hyperplasia and adrenal neoplasms within a similar period (Dickie and Woolley, 1949). In all three groups of mice, the adrenal hyperplasia or neoplasia is followed in a matter of months by growth of accessory reproductive structures. That the abnormal adrenal cortical tissue is the source of the stimulating hormone is inferred from the production of genital development in animals into which *CE* strain adrenal cortical neoplasms are transplanted (Woolley and Little, 1946).

The apparent morphological maturity of the reproductive apparatus together with observations on irregularly cyclic vaginal estrus in *DBA* and in *CE* gonadectomized females (Fekete *et al.*, 1941; Woolley and Little, 1945b) suggested that these gonadectomized females might mate as a result of hormones secreted by their abnormally-developed adrenal cortices.

EXPERIMENTAL PROCEDURE

One 10-month, two 11-month, and five 12-month gonadectomized *DBA* females were followed by vaginal smear (lavage method) for 28 days. Two 10-month gonadectomized *CE* females and two 12-month ovariectomized females from each reciprocal cross were followed for a 20-day period. When these animals showed estrus or estrus-like vaginal smears they were placed with males overnight, and examined the following morning for copulation plugs. As one control, all the experimental females were given at least one trial with the male when the smear indicated a non-estrus condition. As an additional control, six-month *C 57Black* females, gonadectomized soon after birth (without showing subsequent adrenal cortical hyperplasia) were given trials.

At the end of the observation period, all experimental animals were laparotomized or autopsied. No ovarian tissue was found in any animal. All *DBA*'s had marked bilateral adrenal cortical hyperplasia, and all *CE*'s and hybrids showed adrenal neoplasms.

RESULTS

Vaginal cycles were very irregular, occurring at intervals of 1-24 days, and estrus periods were variable in duration (1–11 days). Sharp fluctuation in cellular pattern from one phase of the cycle to another was not seen. Therefore the criterion for estrus was taken to be substantial increase in the ratio of cornified cells to leukocytes.

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In general, plugs were found only on days following the occurrence of estrus-like smears. Eighty-seven matings were set up. Of these, 63 were tried after estrus smears. Mating (as evidenced by plugs) occurred in 23 (36.5%). In the control series, 24 trials were run following non-estrus smears; only one of these resulted in a mating. In the *C57 Black* control series there were no matings, the vaginas in these animals being either closed or only minimally open.

Mating, when occurring, appeared to correlate with vaginal estrus periods. One difference of interest was noted between the *DBA* (adrenal hyperplasia) and *CE* and hybrid (adrenal neoplasms) estrus females: the former group mated 9 times in 36 trials (25%), the latter 14 times in 27 trials (51.8%), which suggests that the neoplastic adrenal cortex may prepare the animal for mating better than the hyperplastic gland.

The female mating response to the male was typical in all respects in the cases observed.

SUMMARY

Gonadectomized female mice of the *DBA* and *CE* strains and reciprocal F_1 hybrids with adrenal cortical hyperplasia or neoplasms exhibit irregular estrous cycles, as evidenced by vaginal smear.

Either adrenal abnormality enables gonadectomized females to mate in a normal fashion, in general, when the vaginal smear indicates an estrus condition, and not otherwise.

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CRAVING FOR POTASSIUM AFTER THE ADMINISTRATION OF ALLOXAN

In carrying out experiments on dietary selection by alloxan diabetic rats (Vartiainen and Bastman-Heiskanen, 1947) the authors in several cases observed in the animals a strong craving for potassium following the administration of the alloxan.

METHODS

The rats were kept in individual cages and allowed to make self-selection of their food from solids and fluids according to Richter's (1938, 1941) method. The adequacy of the food offered was checked by keeping normal control rats under identical test conditions for one year. Potassium chloride was offered in 1 per cent solution. The amount of each foodstuff ingested and the body weight were recorded twice weekly. Determination of the blood sugar level was usually made weekly by the Hagedorn-Jensen method. The alloxan was administered in 5 per cent solution as a subcutaneous injection.

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RESULTS

Table 1 shows the intake of potassium chloride under the different test conditions. Prior to the administration of alloxan the rats in all the test groups were kept for some weeks on a self-selection diet ("Normal"). "Stabilized diabetes" indicates diabetes of not less than two weeks' duration. After stabilization of the diabetes the animals in group IV were kept on alternating insulin and insulin-free periods of a few days. Alloxan was administered to the group V rats in dosages which were insufficient to cause manifest diabetes or increased blood sugar levels. The number of injections of alloxan varied from

TABLE 1. AVERAGE DAILY INTAKE OF POTASSIUM CHLORIDE MG. PER KG. BODY WEIGHT

	Number of animals	Length of period, days	Blood sugar, mg. per 100 cc.	Body weight, grams	Insulin per animal, units	Intake of potassium chloride, g./Kg.
Group I. Normal Controls	3	355	117	348	—	0.040
Group II. Normal	31	25	116	302	—	0.040
Fresh Diabetes, with Insulin	31	9	446	268	1	0.220
Group III. Normal	10	25	121	287	—	0.050
Stabilized Diabetes	10	15	376	260	—	0.190
Group IV. Normal	10	25	119	314	—	0.040
Stabilized Diabetes	10	24	424	266	—	0.150
Stabilized Diabetes, with Insulin	10	22	420	266	1	0.080
Group V. Normal	8	61	123	269	—	0.050
Alloxan* in Repeated Doses, without Diabetes	8	37	136	274	—	0.220

* Total of alloxan per animal averaged 685 mg. per kilogram body weight.

3 to 13, the individual dosages from 50 to 200 mg. per kilogram of body weight, and the total dosages from 440 to 1,100 mg. (mean 685 mg.) per kilogram of body weight. The period of injection lasted from 13 to 133 days.

The potassium intake of the normal rats was approximately equal in the different groups (40–50 mg. per kilogram of body weight per day). Alloxan diabetes brought about a manifold increase in this intake, especially in fresh cases of the disease (group II). In stabilized cases slightly smaller amounts were ingested (groups III and IV). The daily administration of zinc protamine insulin resulted in a reduced need for potassium (group IV). It deserves mention that a strong craving for potassium was also manifested in rats which did not develop diabetes regardless of several injections of alloxan (group V).

Fig. 1 shows examples of some cases in which there was an acute and transient increase in the potassium intake immediately or comparatively soon after the administration of alloxan. The blood sugar level varied in these cases between 300 and 1000 mg. per 100 cc. on the first few days following the injection.

Fig. 2 shows five cases where several injections of alloxan were given but no increase occurred in the blood sugar level. The development of the craving for potassium is to be distinctly seen.

DISCUSSION

The craving for potassium which was manifested in these experiments appeared to be in the main a transient reaction which followed the administration of alloxan and was not necessarily associated with increased blood sugar. In some cases the amount of potassium consumed by the animals was very high. On the other hand, an already sta-

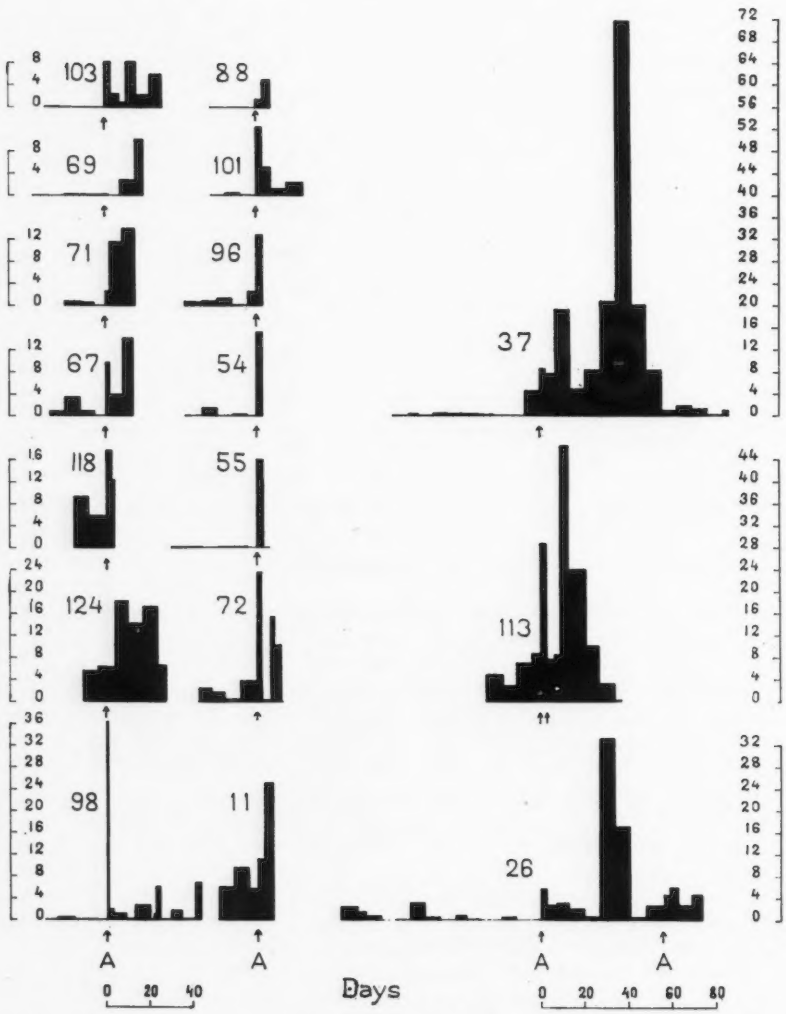

FIG. 1. Craving for potassium after one or two subcutaneous injections of alloxan. All the rats showed high blood sugar levels after alloxan. Ordinate: intake of potassium chloride, cubic centimeters of a one per cent solution. A = alloxan. The arrows indicate the alloxan injections.

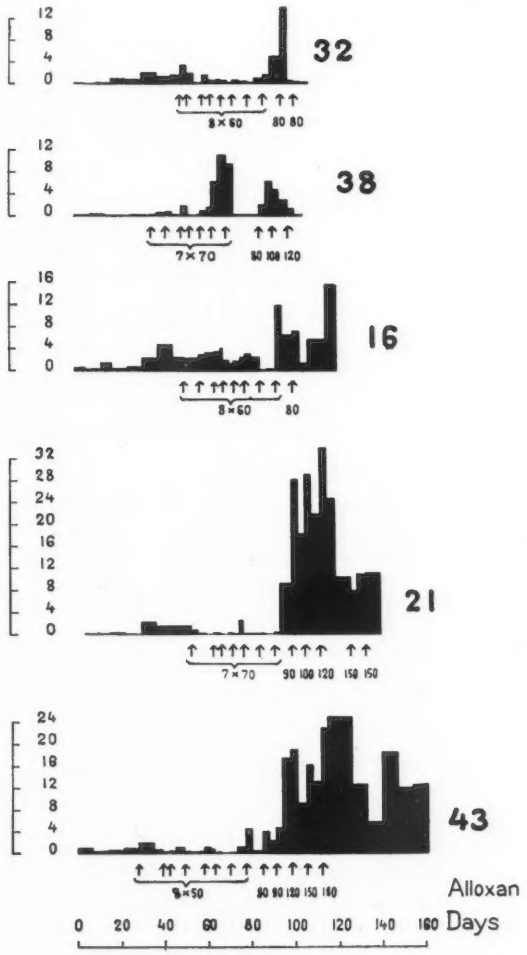

FIG. 2. Craving for potassium after repeated subcutaneous injections of alloxan. All these rats showed normal blood sugar levels after alloxan. Ordinate: intake of potassium chloride, cubic centimeters of a one per cent solution. Dose of alloxan, mg. per kg. of body weight. The arrows indicate the alloxan injections.

bilized alloxan diabetes of long duration was accompanied by an increased need for potassium, which could be reduced by the administration of insulin. In the experiments carried out by us in 1941 no increase in the potassium intake was found following pancreatectomy; the same finding was made in similar experiments by Richter *et al.* (1945). A difference is therefore observed in this respect between the two experimental diabetic conditions.

The increased need for potassium should be regarded principally as a compensatory protective measure. The alloxan apparently exerts a strong stimulus upon the adrenal cortex, which greatly interferes with the potassium metabolism of the body.

The results obtained in our experiments are closely related to the recent clinical observations that the use of potassium is of benefit in diabetic coma.

SUMMARY

The rats used in these experiments were kept in individual cages on an adequate self-selection diet.

Following the injection of alloxan the animals frequently consumed considerably larger amounts of potassium than prior to the injection. Stabilized diabetes was also associated with an increased need for potassium, which was reduced by the administration of insulin.

Such repeated injections of alloxan which did not cause diabetes developed a strong craving for potassium in the animals.

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VARTIAINEN, I., AND L. BASTMAN-HEISKANEN: *Ann. Med. Int. Fenn.* **36**: 740. 1947.

INCREASED APPETITE FOR PROTEIN AFTER THE ADMINISTRATION OF ALLOXAN

The authors have carried out experiments on changes in the dietary selection of rats rendered diabetic by the administration of alloxan.

METHODS

The rats were kept in individual cages and allowed to choose their food from simple foodstuffs (sucrose, casein, olive oil, and yeast) and saline solutions (Richter *et al.*, 1938; Vartiainen and Bastman-Heiskanen, 1947). After a control period of some weeks (indicated in the Figures as "Normal Rats") the animals were given a subcutaneous injection of 80–200 mg. of alloxan per kilogram of body weight in 5 per cent solution. Changes in dietary selection were observed during the following two weeks ("Fresh

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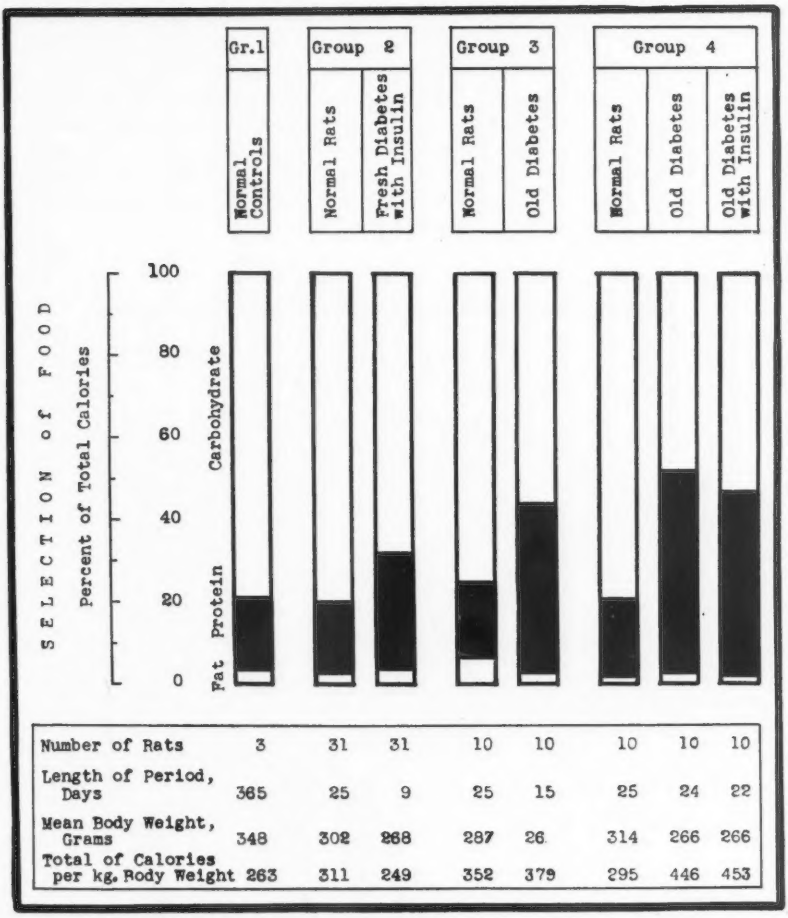


Number of Rats	3	31	31	10	10	10	10	10
Length of Period, Days	365	25	9	25	15	25	24	22
Mean Body Weight, Grams	348	302	268	287	26	314	266	266
Total of Calories per kg. Body Weight	263	311	249	352	379	295	446	453

FIG. 1. Dietary selections by four groups of rats, expressed in percentages of the total number of calories. Lower white segment: fat. Black segment: protein. Upper white segment: carbohydrate.

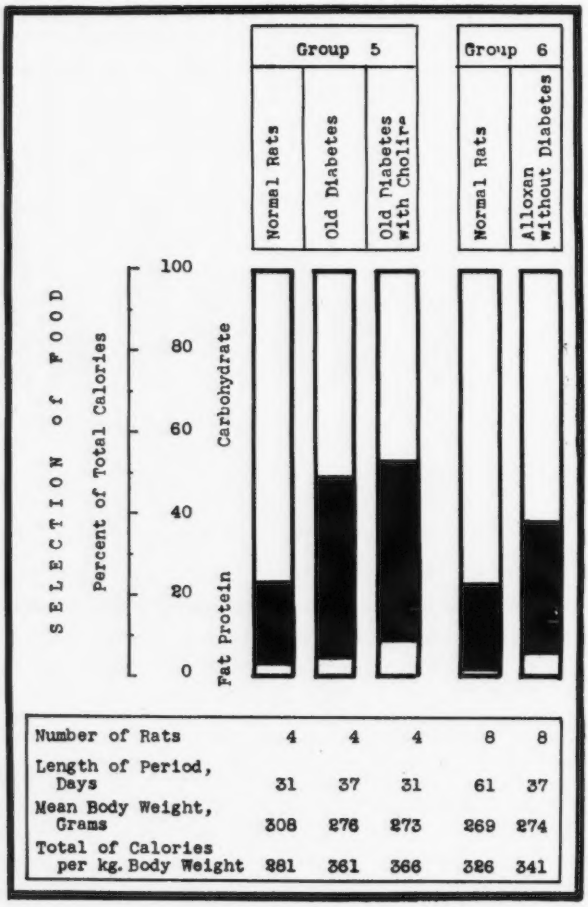
Diabetes") or later ("Old Diabetes"). The insulin used was the protamine zinc variety and was administered subcutaneously in dosages of 1 unit per rat per day. Choline was also given subcutaneously in daily dosages of 300 mg. of choline hydrochloride per kg. body weight in 1 per cent solution. The rats in group 6 received alloxan in several dosages which were insufficient to cause the diabetic syndrome. The number of administrations varied from 3 to 13, the individual dosages from 50 to 200 mg. per kg. body weight, and the total dosages from 440 to 1,100 mg. per kg. body weight. The injections were given over 13 to 133 days. In calculating calories it has been regarded, in accordance with Frey (1930), that dried yeast consists of approximately one-half protein and one-third carbohydrates.

RESULTS

The results are shown in Figs. 1 and 2.

Group 1 comprised the **normal control rats,** which were kept on a self-selection diet for one year. They were in good health, thus indicating the adequacy of the diet selected by them. As regards the percentile proportion of the different sources of calories in their diet, the major part of their calorie requirement was satisfied by carbohydrates. Protein comprised about one-fifth of the total calories. Fat was of very slight significance. It deserves to be noted that the calorie selection was similar in the other groups of rats prior to the administration of alloxan ("Normal Rats").

Fresh diabetes (group 2) caused a reduction in the ingestion of carbohydrates. The



Number of Rats	4	4	4	8	8
Length of Period, Days	31	37	31	61	37
Mean Body Weight, Grams	308	276	273	269	274
Total of Calories per kg. Body Weight	881	361	366	326	341

FIG. 2. Dietary selections by two groups of rats, expressed in percentages of the total number of calories. Column colors as in Fig. 1.

increase in the protein intake was mostly relative and due to the decrease in the total number of calories (primarily of carbohydrates).

Old diabetes designates a diabetic condition of not less than two weeks' duration, in the course of which the symptoms had already become stabilized. In this condition the proportion of carbohydrates in the diet was greatly reduced and the amount of protein had increased correspondingly (groups 3, 4 and 5).

The administration of **insulin** in old diabetes (group 4) effected but slight change in the dietary selection. The ingestion of protein declined somewhat.

The administration of **choline** (group 5) hardly affected the selection of foodstuffs by the rats.

When **alloxan** was administered a number of times in dosages which were not sufficient to cause diabetes (group 6) the rats manifested the diabetic choice of diet.

DISCUSSION

The decrease in the carbohydrate intake and increase in the protein intake of the alloxan diabetic rats observed by the authors is to be undoubtedly regarded as an effort to relieve the disturbed metabolism. This is supported by the fact that when the rats were prevented from selecting their diet more difficult diabetic symptoms were seen in the animals. However, they were not able to fully correct the diabetic symptoms with the aid of their dietary selection. Blood sugar determinations made in the morning revealed very high values (300–400 mg. per 100 cc. blood) and the total intake of water by rats with old diabetes was from four to eight times as great as that of normal rats. In fresh diabetes only about twice as much water was ingested as by the normal rats.

It is interesting to make comparisons in this connection between the results obtained by the authors in alloxan diabetes and the dietary selection of Richter's rats after subtotal pancreatectomy. In their earlier report Richter *et al.* (1941) state that following the operation the rats decreased their ingestion of carbohydrates and increased that of fat, whereas in a later experiment by Richter and coworkers (1945) the test rats consumed a smaller amount of carbohydrates and a greater amount of proteins. When allowed to make their own choice of diet the rats also consumed less water. Unfortunately no blood sugar determinations were made by Richter. This mild diabetic condition following subtotal pancreatectomy probably corresponds to those rats in the present study which were given alloxan in several injections but in dosages which were too small to cause manifest diabetes and increased blood sugar levels, even though there was a transition to the diabetic selection of food and the ingestion of water almost doubled.

It is interesting, furthermore, to note that the insulin dosage used by us was able only slightly to adjust the selection of foodstuffs in a normal direction.

SUMMARY

Rats were kept in individual cages in which they were allowed to make self-selection from simple foodstuffs.

The total number of calories consumed by the normal rats consisted of from 18 to 21 per cent of proteins and from 75 to 80 per cent of carbohydrates. Fat played a less significant part in the diet. With rats with alloxan diabetes of not less than two weeks' duration, protein comprised from 42 to 50 per cent and carbohydrates from 48 to 56 per cent of the total number of calories ingested.

The changes in the dietary selection effected by insulin and choline were of minor significance.

When alloxan was administered in several dosages which were insufficient to cause

manifest diabetes the protein intake increased from 21 to 33 per cent and the carbohydrate intake declined from 78 to 62 per cent.

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NEW BOOK

PRACTICAL NEUROLOGICAL DIAGNOSIS. 4th Edition. By R. Glen Spurling. Published by Charles C Thomas, Springfield, Ill. 1950. xx+268 pp. Price $5.00.

This manual is designed "to present a simple account of the principles of neurological diagnosis." Because of its simplicity, the reviewer feels that its usefulness is extremely limited. It is much too elementary to give the student or practitioner anything but a smattering of neurological knowledge. Since he would need a standard neurological text in addition, one wonders at the wisdom of investing in this rather inadequate handbook.

The volume requires a ruthless editing. For example, it is stated that during a convulsion cortical cells are excited "into rhythmic discharge which persists until the threshold (sic) is lowered through exhaustion." Obviously the author intended to say "until the *excitability* is lowered. . . . "

Even the neuro-anatomy is confusing. What is meant when, during the discussion of the 6th nerve, it is stated that "Its nucleus lies in the dorsal portion of the pons just posterior to the facial nucleus."?

At one point the figures for the protein and glucose contents of the cerebrospinal fluid are given incorrectly. Several pages later they are given correctly.

Certain other more general criticisms could be made. The role of the Betz cells in giving rise to the pyramidal tract is over-emphasized. The description of the physiology of the hypothalamus in regard to temperature regulation is inaccurate. Finally the discussion of the anatomy and physiology of the basal ganglia could be brought much more up to date.

This book can be recommended only to those who desire a quick, simple, and occasionally incorrect view of a very complicated field.

PAUL O. CHATFIELD, M.D.